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TESIS DOCTORAL

Desarrollo de nuevas metodologías avanzadas para el análisis de oligosacáridos bioactivos

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PRESENTADA POR

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Mención internacional

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A mis padres…

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"La raíz de todo bien reposa en la tierra de la gratitud y hace que el corazón hable", Dalai Lama.

Un impulso innato del ser humano como especie, a lo largo de la historia, ha sido el de buscar una nueva frontera, horizonte o entorno, y superarlo, para así progresar.

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RESUMEN/SUMMARY

La ciencia no es solo una disciplina de razón
sino también de romance y pasión.

Stephen Hawking

RESUMEN

En la presente memoria se exponen los resultados más relevantes del trabajo titulado "Desarrollo de nuevas metodologías avanzadas para el análisis de oligosacáridos bioactivos". En dicho trabajo se han optimizado, validado y aplicado diferentes métodos analíticos basados en cromatografía de gases (GC) y de líquidos (LC), acopladas a espectrometría de masas (MS), con el fin de superar muchas de las limitaciones asociadas a las metodologías hoy en día en uso para el análisis de oligosacáridos bioactivos, destacando los oligosacáridos de leche de cabra (COS).

La leche de cabra es una fuente rica de carbohidratos, mayoritariamente constituida por lactosa, pero también por varios oligosacáridos bioactivos y de especial interés. El análisis de estos oligosacáridos no es una tarea sencilla y normalmente requiere de un paso previo de fraccionamiento para eliminar la lactosa. Para ello, en esta tesis, se han empleado técnicas biotecnológicas basadas en el uso combinado de enzimas con actividad β-galactosidasa que hidrolicen la lactosa pero conserven el perfil de COS y de levaduras que eliminen los productos de hidrólisis producidos. Otra técnica empleada en esta tesis para el fraccionamiento de la lactosa ha sido la cromatografía de exclusión molecular (SEC) que permite la separación de los oligosacáridos por su grado de polimerización.

Gracias a estos métodos de fraccionamiento, los COS han sido identificados y caracterizados mediante técnicas cromatográficas. Dentro de ellas, la GC-MS es una técnica ampliamente utilizada para el análisis de carbohidratos de bajo peso molecular debido a su alta resolución, eficiencia y capacidad de identificación, sin embargo, es necesaria una etapa previa de derivatización de la muestra. Para ello, en esta tesis se han optimizado las condiciones de derivatización (tiempo, temperatura y reactivo de sililación) de diferentes patrones de carbohidratos. El método desarrollado se ha aplicado de manera exitosa a una muestra de leche de cabra.

La LC es otra de las técnicas más empleadas por su alta versatilidad y múltiples modos de operación. En esta tesis se combina el empleo de la cromatografía líquida con columna de nano-chip acoplada a espectrometría de masas con analizador de cuadrupolo-tiempo de vuelo (nano-LC-Chip-Q-ToF MS) para la identificación de 78

oligosacáridos de calostro de cabra y la cromatografía de interacción hidrofílica acoplada a espectrometría de masa con analizador de cuadrupolo (HILIC-Q MS) para la cuantificación de los mayoritarios. Esta combinación se empleó para llevar a cabo por primera vez un estudio exhaustivo de la evolución de los COS, tanto neutros como ácidos, durante diferentes etapas de lactación.

Por otro lado, en esta tesis la cromatografía de líquidos también se ha aplicado para el análisis de carbohidratos con distintas unidades monoméricas, grados de polimerización y enlaces glicosídicos, combinando diferentes modos de operación como la HILIC y la cromatografía de carbón grafitizado (GCC), para estudiar el comportamiento cromatográfico de disacáridos y técnicas espectrométricas como MS en tándem (MS^n). Así se ha conseguido establecer un perfil estructural completo y se ha podido caracterizar trisacáridos con diferentes estructuras. También se ha desarrollado un método mediante LC×LC para el análisis de estos carbohidratos, derivatizándolos previamente con ácido 4-aminobenzoico (ABEE). Se empleó una combinación ortogonal HILIC × fase inversa (RP) usando una interfase con modulación activa. Este método se aplicó con éxito al análisis, por primera vez, de mezclas complejas de patrones de oligosacáridos y de mezclas comerciales de genti-oligosacáridos y galactooligosacáridos prebióticos.

Los resultados descritos en esta Tesis suponen una contribución destacable en el estudio de carbohidratos bioactivos y en el empleo de las técnicas cromatográficas acopladas a espectrometría de masas.

SUMMARY

In this PhD dissertation the most relevant results of the work entitled "Development of new advanced methodologies for the analysis of bioactive oligosaccharides" are presented. In this work, different analytical methods based on gas (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) have been optimized, validated and applied to bioactive oligosaccharides, in particular to goat milk oligosaccharides (COS), in order to overcome nowadays limitations associated with their analysis.

Goat milk is a rich source of carbohydrates, mainly constituted by lactose, but also by several bioactive oligosaccharides of special interest. However, their analysis is not straightforward and requires a previous fractionation step to remove lactose present at high concentration; therefore, in this thesis a biotechnological methodology based on the combined used of β-galactosidases to hydrolyse lactose but preserving the COS profile and yeasts to remove the hydrolysis products, has been used. Moreover, size exclusion chromatography (SEC) has also been used as a fractionation technique due to the good separation provided in terms of degrees of polymerization.

After fractionation, COS have been identified and characterized by chromatographic techniques. Among them, GC-MS is widely used for the analysis of low molecular weight carbohydrates due to its high resolution, efficiency and identification capacity. However, a previous derivatization step is mandatory. For this purpose, in this thesis different conditions such as time, temperature and silylation reagents for derivatization of carbohydrate standards have been optimized and successfully applied to the GC-MS analysis of a goat milk sample.

LC is also a widely used technique for carbohydrate analysis due to its high versatility and multiple operation modes. In this thesis, the combined use of nano-Chip liquid chromatography coupled to mass spectrometry with quadrupole time of flight analyser (nano-LC-Chip-Q-ToF-MS) to identify up to 78 goat colostrum oligosaccharides and hydrophilic interaction liquid chromatography coupled to mass spectrometry with quadrupole analyser (HILIC-Q MS) to quantify the main ones, has been proposed. This combination has been successfully applied for the comprehensive study of goat milk oligosaccharides, both neutral and acidic, at different lactation stages, for the first time.

On the other hand, in this thesis LC has been also applied to the analysis of carbohydrates with different monomeric units, degrees of polimerization and glycosidic linkages. The correlation of chromatographic retention data such as HILIC and graphitized carbon chromatography (GCC) and characteristic fragment ions of disaccharides observed by tandem MS (MS^n) with statistical analysis was successful in establishing some specific criteria that allowed the characterization of trisaccharides with different structural features. Moreover, a LC×LC method has been developed for analysis of di- and trisaccharides, previously derivatized by 4-aminobenzoic acid (ABEE). An orthogonal combination of HILIC × reverse phase (RP) using an active modulation interface was used. This method has been successfully applied, for the first time, to the analysis of complex mixtures of oligosaccharide standards and prebiotic commercial mixtures of galactooligosaccharides and gentiooligosaccharides.

The results presented in this PhD thesis can remarkably contribute to the study of bioactive carbohydrates and to the use of chromatographic coupled to mass spectrometric techniques.

ABREVIATURAS

^{1}D	Primera dimensión
2-AP	2-aminopiridina
^{2}D	Segunda dimensión
2-FL	α-2'-Fucosil-lactosa
$^{2}t_{c}$	Tiempo de retención en la segunda dimensión
3-GL	α-3'-Galactosil-lactosa
3-SHL	3'-Sialil-6'-galactosil-lactosa
3-SL	3'-Sialil-lactosa
6-GL	β-6'-Galactosil-lactosa
6-SL	6'-Sialil-lactosa
6-SHL	6'-Sialil-3'-galactosil-lactosa
A_0	Ortogonalidad
ABEE	4-aminobenzoato de etilo
ACN	Acetonitrilo
APCI	Ionización química a presión atmosférica
BEH	Columna híbrida de puentes de etileno
BSTFA	*N,O*-bis-(trimetilsilil)-trifluoroacetamida
C3	Triosas
C4	Tetrosas
C5	Pentosas
CI	Ionización química
CID	Disociación inducida por colisión
COS	Oligosacáridos de leche de cabra
COSc	Oligosacáridos propios de la leche de cabra

COSg	Galactooligosacáridos presentes en la leche de cabra
COSh	Oligosacáridos formados por la hidrólisis de COS
DNAL	Di-*N*-acetil-glucosaminil-lactosa
DNGHL	Di-*N*-glicolil-neuraminilhexosil-lactosa
DNGL	Di-*N*-glicolil-neuraminil-lactosa
DP	Grado de polimerización
DSL	Disialil-lactosa
EI	Impacto electrónico
ESI	Electronebulización o ionización por electroespray
F	Valor de distribución estadística
FID	Detector de ionización de llama
FOS	Fructooligosacáridos
Fru	Fructosa
Fuc	Fucosa
Gal	Galactosa
GalNAc	*N*-acetilgalactosamina
GC	Cromatografía de gases
GC×GC	Cromatografía de gases bidimensional completa
GCC	Cromatografía de carbón grafitizado
GC-GC	Cromatografía de gases multidimensional clásica
GC-MS	Cromatografía de gases acoplada a espectrometría de masas
GEOS	Gentioligosacáridos
Glc	Glucosa
GlcNAc	*N*-acetilglucosamina
GOS	Galactooligosacáridos
GRAS	Generalmente reconocido como seguro

HETP	Altura equivalente de plato teórico
HILIC	Cromatografía de interacción hidrofílica
HMDS	Hexametildisilazano
HMOs	Oligosacáridos de la leche humana
HPAEC	Cromatografía de alta eficacia de intercambio aniónico
HPCEC	Cromatografía de alta eficacia de intercambio catiónico
IEC	Cromatografía de intercambio iónico
IT	Trampa iónica
LC	Cromatografía de líquidos
LC×LC	Cromatografía de líquidos bidimensional completa
LC-LC	Cromatografía de líquidos multidimensional clásica
LMWC	Carbohidratos de bajo peso molecular
LNFP III	Lacto-*N*-fuco-pentaosa III
LNFP V	Lacto-*N*-fuco-pentaosa V
LNH	Lacto-*N*-hexaosa
Man	Manosa
MD-LC	Cromatografía de líquidos multidimensional
MeCN	Acetonitrilo
MSTFA	*N*-metil-*N*-(trimetilsilil)trifluoroacetamida
NADHL	*N*-Acetil-glucosaminildihexosil-lactosa
NAHL	*N*-Acetil-glucosaminilhexosil-lactosa
NAL	6'-*N*-Acetil-glucosaminil-lactosa
NALNH	*N*-Acetil-glucosaminil-lacto-*N*-hexaosa
n_c	Capacidad de pico
NCE%	Energía de colisión normalizada
Neu5Ac	Ácido *N*-acetil neuraminico

Neu5Gc	Ácido *N*-glicolil neuraminico
NGL	6'-Glicolil-neuraminil-lactosa
NMR	Renonancia magnética nuclear
NPLC	Cromatografía de fase normal
PABA	4-aminobenzoato de etilo
PAD	Detector de amperometría de pulsos
PGC	Columna de carbón grafitizado
PLE	Extracción con líquidos presurizados
Q	Cuadrupolo simple
QqQ	Triple cuadrupolo
Q-ToF	Cuadrupolo acoplado a tiempo de vuelo
RF	Factor de respuesta
RI	Índice de refracción
RPLC	Cromatografía en fase inversa
R_s	Resolución
SDHL	Sialil-di-hexosil-lactosa
SEC	Cromatografía de exclusión molecular
SFE	Fluidos supercríticos
SNAL	Sialil-*N*-acetilglucosaminillactosa
SNGHL	Sialil-*N*-glicolil-neuraminilhexosil-lactosa
SNGL	Sialil-*N*-glicolil-neuraminil-lactosa
SNLH	Sialil-lacto-*N*-hexaosa
SPE	Extracción en fase sólida
TFA	Ácido trifluoroacético
t_g	Tiempo de gradiente
TMCS	Trimetilclorosilano

TMS	Trimetilsilil derivados
TMSI	Trimetilsililimidazol
TMSO	Trimetilsilil oximas
ToF	Analizador de tiempo de vuelo
t_R	Tiempo de retención
UV	Ultravioleta
w_b	Anchura de pico en la base
w_h	Anchura de pico a mitad de altura

1. INTRODUCCIÓN

Lo bueno de la ciencia es que es cierta, creas o no en ella.

Neil DeGrasse Tyson

1. INTRODUCCIÓN

1.1 Carbohidratos en alimentos

Los carbohidratos son uno de los constituyentes más importantes de los alimentos. Pueden estar presentes de manera natural o añadirse para la mejora de sus características fisicoquímicas, calidad nutricional y/o organoléptica, propiedades funcionales, etc. Se caracterizan por su gran diversidad estructural y el alto número de isómeros que presentan.

En función de su tamaño, los carbohidratos se pueden clasificar en monosacáridos, oligosacáridos y polisacáridos. Los **monosacáridos** son las unidades más pequeñas y están compuestos por una cadena de átomos de carbono a los que se unen distintos grupos hidroxilo. Dependiendo del número de átomos de carbono se denominan triosas (C3), tetrosas (C4), pentosas (C5), etc. y según la naturaleza de su grupo carbonilo, si está en posición terminal o no, pueden ser aldosas o cetosas (Biermann, 1989; Collins y Ferrier, 1995). En soluciones acuosas, la mayoría de los monosacáridos, especialmente los constituidos por 5 y 6 átomos de carbono, pueden encontrarse en forma cíclica (hemiacetal), como resultado de la reacción del grupo carbonilo con uno de los grupos hidroxilos de la molécula, siendo del tipo furanosa (anillos de 5 elementos) o piranosa (anillos de 6 elementos). Tras la ciclación, el carbono anomérico permite dos conformaciones en función de la orientación del grupo hidroxilo, la α y la β. Si este grupo hidroxilo se encuentra libre, se denomina azúcar reductor, mientras que si está bloqueado, es no reductor. Los que son reductores, cuando se encuentran en disolución, pueden presentar hasta 5 formas tautoméricas diferentes en equilibrio (Figura 1.1): α y β furanosas, α y β piranosas y cadena abierta, estando las cíclicas más favorecidas (Grindley, 2008).

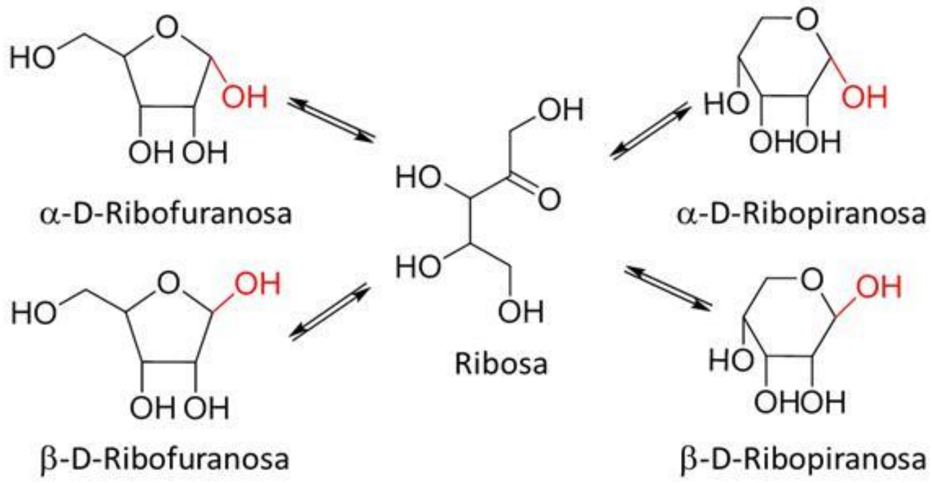

Figura 1.1: Representación de las 5 formas tautoméricas posibles de un carbohidrato tomando ribosa como modelo.

Los **oligosacáridos** están constituidos por varias unidades de monosacáridos unidos por enlaces *O*-glicosídicos. En dichos enlaces reacciona el grupo hidroxilo del carbono anomérico del primer monosacárido con un grupo hidroxilo unido a un carbono (anomérico o no) del segundo monosacárido, con pérdida de una molécula de agua. Debido a que cada monómero puede unirse a otros mediante más de un enlace, pueden formarse estructuras ramificadas. Dependiendo del número de unidades, se denominan disacáridos, trisacáridos, tetrasacáridos, etc., siendo los más abundantes en los alimentos los disacáridos como la lactosa, maltosa y sacarosa.

Los **polisacáridos** son macromoléculas constituidas por un gran número de monosacáridos formando cadenas lineales o ramificadas. El almidón y la celulosa son algunos de los polisacáridos más comunes en los alimentos.

No existe una regla estricta de clasificación entre oligosacáridos y polisacáridos. El término "oligosacárido" hace referencia a una molécula con estructura definida (generalmente hasta 10 ó 20 unidades) en contraposición al de "polisacárido" que suele hacer referencia a una macromolécula de longitud no especificada. Por otro lado, el término "azúcar" se aplica a monosacáridos y oligosacáridos de bajo peso molecular. (IUPAC, 1996).

Dentro del término genérico “carbohidrato” se incluyen además otras sustancias obtenidas tanto por reducción de los grupos carbonilo de los azúcares (alditoles y ciclitoles), como por oxidación de uno o más grupos hidroxilo a ácidos carboxílicos, o por sustitución de uno o más grupos hidroxilos por átomos de hidrógeno, grupos amino, grupos tioles o grupos similares heteroatómicos. También dicho término incluye a los derivados de estos compuestos (IUPAC, 1996).

1.2 Propiedades de los carbohidratos como ingredientes alimentarios

Los carbohidratos presentan un gran número de propiedades tanto fisicoquímicas como biológicas que se aprovechan en la elaboración de los alimentos y/o que afectan a la salud del consumidor. Algunas de las principales propiedades de los carbohidratos son:

1.2.1 Propiedades fisicoquímicas

La **higroscopicidad** es la capacidad de los azúcares para absorber el agua de la atmósfera y varía dependiendo de su estructura, de la mezcla de isómeros y de su pureza. Esta característica puede ser deseada, ya que mantiene la humedad en productos con alto porcentaje de azúcar, o por el contrario, puede suponer un problema si se pretende evitar la aglomeración de productos en forma de polvo (Belitz y col., 2009).

La **solubilidad** de carbohidratos de bajo peso molecular es muy alta en medios acuosos y disolventes polares como piridina, dimetilsulfóxido y dimetilformamida, mientras que presentan baja solubilidad en etanol y son insolubles en disolventes orgánicos como cloroformo, éter o benceno. Los polisacáridos son generalmente insolubles o poco solubles en agua (Belitz y col., 2009).

Otra característica de los azúcares es su capacidad de formar **cristales**. Los carbohidratos pueden permanecer en estado no cristalino (estado amorfo) como en jarabes o almíbar de fruta, o en estado vítreo como en los caramelos duros (Chung y col., 1999). En algunos alimentos la cristalización es un proceso no deseado, tal es el caso de la lactosa en leche condensada o helados.

Los carbohidratos presentan muy baja **estabilidad térmica**, ya que se deshidratan con facilidad y a temperaturas elevadas se degradan, produciendo reacciones de caramelización (Claude y Ubbink, 2006). Esta reacción es deseada en muchos procesos de elaboración de alimentos, tales como caramelos, derivados de panadería, leche condensada, etc. ya que proporciona un color y sabor agradable. Sin embargo, es importante controlar el proceso para evitar la formación de sustancias no deseadas que alteren las propiedades organolépticas.

Los carbohidratos son compuestos asociados al **sabor dulce**. La sacarosa destaca entre los demás por su sabor especialmente agradable, incluso en elevadas concentraciones, siendo uno de los edulcorantes más usados. Sin embargo, existen algunos oligosacáridos que son amargos, como la gentiobiosa. En general, el dulzor disminuye conforme aumenta la longitud de la cadena (Belitz y col., 2009).

1.2.2 Propiedades biológicas

Los carbohidratos, son la principal fuente de energía para la mayoría de las células, como es el caso del almidón en plantas y el glucógeno en animales y poseen funciones estructurales, siendo los constituyentes principales de las paredes celulares de los vegetales o del exoesqueleto de muchos artrópodos (Jéquier, 1994). Además, está descrito que los carbohidratos pueden ser moduladores del nivel de glucosa e insulina en sangre, participar en la disminución del colesterol en sangre y en la síntesis de vitaminas del grupo B, favorecer la absorción del calcio, etc. (Crapo, 1996; Sako 1999 y Perugino, 2004).

Asimismo, algunos carbohidratos, como el *myo*-inositol, desempeñan funciones esenciales en la utilización de la grasa, como promotores del crecimiento y exhiben cierta habilidad para mejorar la conductancia nerviosa en diabéticos (Zhao y col., 2018). También se ha descrito que algunos carbohidratos como el *chiro*-inositol (Ostlunf y Sherman, 1996) ayudan a mejorar la ovulación, los parámetros metabólicos y la sensibilidad a la insulina de mujeres con síndrome de ovario poliquístico (Langana y col. 2018). Algunos iminoazúcares son reconocidos inhibidores de glicosidasas, pudiéndose emplear dichos carbohidratos para el tratamiento de numerosas enfermedades relacionadas con la absorción de azúcares, tales como diabetes y obesidad (Rodríguez-Sánchez y col. 2001).

Otra propiedad de los carbohidratos que está cobrando gran importancia en la actualidad es la actividad prebiótica que presentan algunos de ellos. El término **prebiótico** fue establecido en 1995 por Gibson y Roberfroid quienes lo definieron como "un ingrediente alimentario no digerible que afecta beneficiosamente al hospedador al estimular selectivamente el crecimiento y/o actividad de uno o un limitado número de especies bacterianas en el colon, y que por lo tanto mejora la salud". Posteriormente, Roberfroid y col., (2010) ampliaron el concepto de prebiótico indicando que "es un ingrediente que produce una estimulación selectiva del crecimiento y/o actividad(es) de uno o de un limitado número de géneros/especies de microorganismos en la microbiota intestinal confiriendo beneficios para la salud del hospedador".

Como consecuencia de la fermentación de los oligosacáridos prebióticos por las bacterias probióticas se han descrito varias implicaciones positivas para la salud del consumidor, tales como la estimulación del sistema inmune, el incremento de la resistencia a la invasión por las bacterias intestinales patógenas, una actividad intestinal mejorada (regulación de la motilidad intestinal, producción de ácidos grasos de cadena corta, prevención de diarrea y estreñimiento), la protección contra el cáncer de colon o el efecto en la biodisponibilidad de los minerales o la disminución de los niveles lipídicos (Rastall, 2010; Roberfroid, 2010). Así, en la actualidad, existe un gran interés en el empleo de carbohidratos prebióticos para modificar la microbiota intestinal, con el fin de mejorar la salud humana (Nedaei y col., 2019; Lopez-Castejon y col., 2019)

Los oligosacáridos prebióticos son carbohidratos con distintos grados de polimerización (DP), principalmente entre 3 y 10 según la IUB-IUPAC (IUB-IUPAC; Joint Commission on Biochemical Nomenclature (JBC) ,1980), aunque también se pueden considerar algunos disacáridos o incluso monosacáridos (Kunz y col., 2000).

Generalmente no son carbohidratos puros, sino que son mezclas de oligosacáridos con distintas estructuras, conteniendo además mono- y disacáridos. Se pueden encontrar de forma natural, principalmente en alimentos de origen vegetal como alcachofas, soja, cebollas, legumbres, etc. y, en menor medida, en alimentos de origen animal, como leche de algunos mamíferos. Pueden también obtenerse de forma sintética, mediante procesos enzimáticos a partir de azúcares sencillos por transglicosilación o degradación de polisacáridos de origen vegetal o microbiano. Actualmente, en Europa existen cuatro tipos de productos con propiedades prebióticas en el mercado: inulina, fructooligosacáridos (FOS), lactulosa y β-galactooligosacáridos (β-GOS) (Figura 1.2).

- ✓ **Inulina**: polisacárido compuesto por monosacáridos de fructosa unidos con enlaces β (1→2) y una glucosa terminal, enlazados de forma lineal, ramificada o cíclica. Está presente de manera natural en muchas plantas comestibles como la achicoria o la alcachofa.
- ✓ **FOS**: oligosacáridos obtenidos por hidrólisis enzimática de la inulina o mediante transfructosilación a partir de sacarosa utilizando β-fructofuronosidasas (EC 3.2.1.26). Se encuentran en frutas, verduras, cereales, etc.
- ✓ **Lactulosa**: disacárido sintético obtenido a través de la isomerización en medio básico de la lactosa constituido por una molécula de galactosa y otra de fructosa unidas por enlace β (1→4).
- ✓ **β-GOS**: mezclas complejas de oligosacáridos basados en unidades de galactosil-galactosas y galactosil-glucosas, obtenidos a partir de lactosa mediante reacciones de hidrólisis y transgalactosilación catalizadas por la acción de β-galactosidasas (EC 3.2.1.23) procedentes de diversos orígenes microbianos (levaduras, hongos, bacterias). Así, se originan mezclas complejas de GOS con enlaces β (1→6), β (1→3), β (1→4), etc. y un grado de polimerización de entre 2 y 6, dependiendo de la fuente de la enzima y de las condiciones de reacción empleadas (Matsumoto y col. 1993).

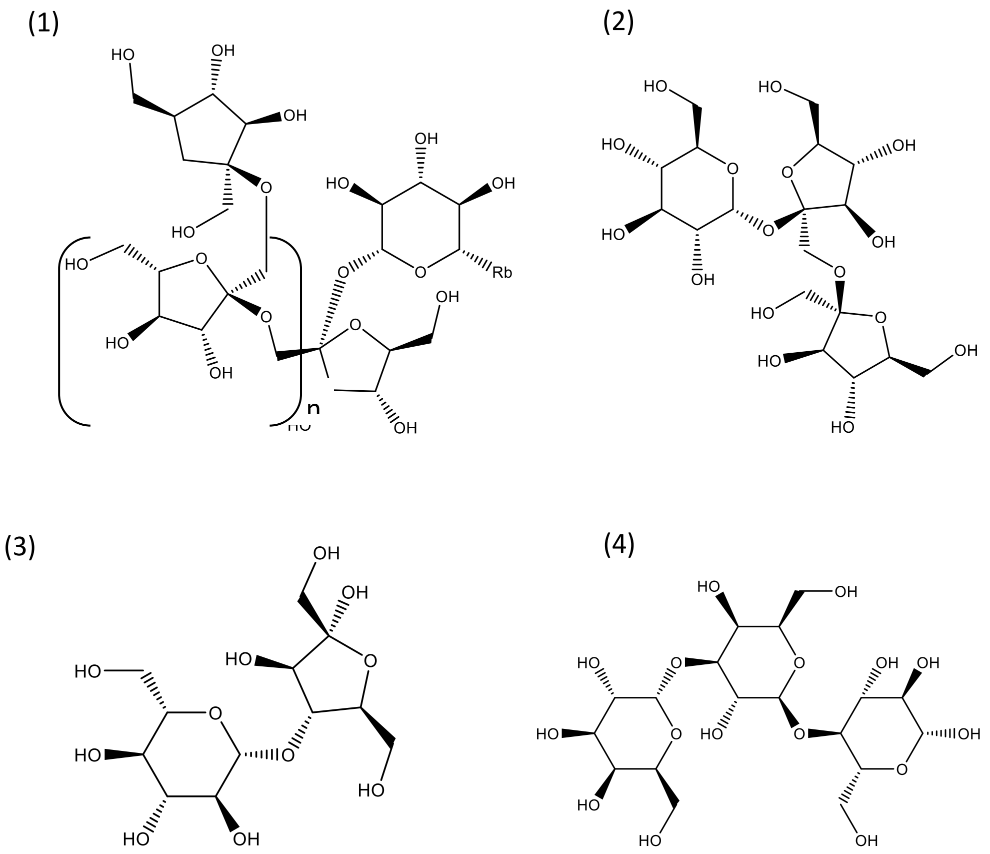

Figura 1.2: Ejemplo de algunos carbohidratos reconocidos como prebióticos:

(1) Inulina, (2) 1-kestosa (FOS), (3) lactulosa y (4) 3’- galactosil-lactosa (GOS).

Sin embargo, en el mercado de Japón se encuentran disponibles éstos y otros oligosacáridos con propiedades prebióticas, tales como isomaltooligosacáridos y gentioligosacáridos (unidades de glucosa unidas por enlace α (1→6) y β (1→6) respectivamente), oligosacáridos de soja (con rafinosa y estaquiosa como principales componentes), lactosacarosa (trisacárido obtenido a partir de lactosa y sacarosa por transfructosilación), y xilooligosacáridos (unidades de xilosa con enlaces β (1→4)).

Existe además un gran interés por la obtención de nuevos carbohidratos que no solo presenten propiedades prebióticas, sino que además posean otras propiedades adicionales beneficiosas para la salud, pudiendo ser ingredientes multifuncionales. En este sentido, destacan los **oligosacáridos de la leche humana (HMOs),** que han sido considerados como los primeros prebióticos, al ser responsables del alto número de bifidobacterias presentes en heces de lactantes (Coppa y col., 2004). Además, los HMO actúan como receptores análogos evitando la adhesión de patógenos al epitelio intestinal (Lane y col., 2010; Bode, 2012), presentan propiedades antiinflamatorias y moduladoras del sistema inmune, ejercen efectos sistémicos, etc. (Guarner, 2009, Chichlowski y col., 2011; Kunz y col., 2014).

Los HMOs están formados por combinaciones de 5 monosacáridos diferentes: glucosa (Glc), galactosa (Gal), fucosa (Fuc), *N*-acetilglucosamina (GlcNAc) y ácido *N*-acetilneuramínico (NeuAc) o ácido siálico y son cadenas que varían desde 3 a 15 unidades de estos carbohidratos (Thomson y col., 2018). La fracción de los HMO es muy compleja, al menos puede estar formada por 1000 componentes (Corzo y col. 2015), que pueden alcanzar en su conjunto concentraciones comprendidas entre 10-15 g L^{-1} (Petherick 2010). Hasta el momento, se han identificado más de 200 HMO, de los cuales solo 80 han podido ser totalmente caracterizados (Bode, 2006; Venema, 2012; Barile y Rastall, 2013).

En la actualidad existe un gran interés por la búsqueda de otras fuentes de oligosácaridos alternativas a los HMOs, que presenten una funcionalidad similar y que puedan ser explotadas a gran escala, bien mediante consumo directo o bien como ingrediente funcional de preparados alimentarios como son las fórmulas infantiles (Boehm y Stahl, 2007; Urakami y col., 2018; Nijman y col., 2018). La gran complejidad de los HMOs dificulta su síntesis, sin embargo, las leches de otros mamíferos, sobre todo los domésticos como la cabra, la oveja o la vaca, constituyen una clara alternativa. Una de las características comunes y más relevantes de los oligosacáridos presentes en estas leches es el enlace β-glicosídico de la galactosa y de la GlcNAc. El intestino humano carece de enzimas capaces de hidrolizar estos enlaces glicosídicos, excepto el de lactosa; de este modo, dicho enlace glicosídico es un elemento estructural común que protege a estos oligosacáridos del proceso digestivo (Moro y col., 2005). Esta resistencia digestiva favorece el uso de estos compuestos como agentes prebióticos.

Sin embargo, existen diferencias tanto a nivel cualitativo como cuantitativo en los oligosacáridos presentes en las distintas leches de mamíferos, lo cual puede influir en su utilización como ingredientes funcionales. Es conocido que la **leche de cabra** contiene oligosacáridos estructuralmente más parecidos a la leche humana que leches procedentes de otros mamíferos (Martínez-Ferez y col., 2006, Meyrand y col., 2013). Además, desde el punto de vista cuantitativo, la leche de cabra presenta niveles elevados (0.25-0.30 g L^{-1}) de oligosacáridos en comparación a los encontrados en la leche de vaca (0.03- 0.6 g L^{-1}) u oveja (0.02-0.04 g L^{-1}), aunque significativamente inferiores a los presentes en la leche humana (12-13 g L^{-1}) (Giorgio y col., 2018). En virtud de ello, la leche de cabra supone una buena alternativa a la leche humana. En este contexto, la producción de leche de cabra ha aumentado a nivel mundial un 108,7 % en el periodo de 1988 a 2013, pasando de 9 millones a 18,5 millones de toneladas, siendo Europa el continente de mayor producción y, más concretamente, países como España, Francia, Holanda y Grecia (FAOSTAT, 2013).

1.3 Análisis de carbohidratos

1.3.1 Fraccionamiento

Como se ha mencionado anteriormente, los oligosacáridos bioactivos, tanto los presentes de forma natural en los alimentos como los obtenidos mediante reacciones de síntesis, se encuentran en forma de mezclas complejas constituidas por carbohidratos con estructuras químicas muy similares y presentes en muy diversa concentración. De forma general, los oligosacáridos se encuentran acompañados de mono- y disacáridos en alta concentración. Este alto contenido en azúcares dificulta mucho la caracterización de los carbohidratos minoritarios, por lo que la introducción de una etapa previa de fraccionamiento puede ser de gran utilidad para su análisis. Además, la eliminación de estos carbohidratos de bajo peso molecular, en algunos casos, permitiría obtener una fracción enriquecida en oligosacáridos bioactivos para su potencial uso como ingrediente alimentario. Por tanto, el empleo de métodos de fraccionamiento de carbohidratos presenta un gran interés. Sin embargo, debido a la similitud de estructuras y a la diferencia de concentraciones anteriormente comentadas, no es una tarea sencilla. La búsqueda de métodos de fraccionamiento que ofrezcan alta selectividad y eficacia, empleando pequeños volúmenes de disolvente, respetuosos con el medio ambiente y económicos, es uno de los principales objetivos de los investigadores y la industria alimentaria (Moreno y col., 2014).

Los métodos de fraccionamiento de carbohidratos existentes hasta el momento se basan en: (i) el empleo de membranas (ultra y nano filtración), (ii) técnicas cromatográficas (i.e. cromatografía en columna con carbón activo, cromatografía de exclusión molecular (SEC), cromatografía de intercambio iónico), (iii) empleo de disolventes (mezclas hidroalcohólicas, líquidos iónicos, extracción con líquidos presurizados (PLE), etc.) y (iv) tratamientos microbiológicos o enzimáticos.

Los procesos de **separación con membranas** han sido muy utilizados en un gran número de aplicaciones en la industria alimentaria, debido a su simplicidad y eficacia de operación (Tundis col., 2018). Una membrana puede actuar como filtro selectivo, a veces limitando la difusión entre dos soluciones o como una membrana activa en el que su estructura química determina la selectividad de transferencia de la muestra (Gilar y col. 2001). Las técnicas de ultra y nanofiltración han sido ampliamente usadas en el estudio de carbohidratos, no solo porque permiten el fraccionamiento de éstos, sino también su purificación y concentración (Moreno y col., 2014; Aquino y col., 2017). En este sentido, se ha utilizado la ultrafiltración tanto para la purificación de oligosacáridos de diferentes orígenes, tales como pectooligosacáridos (Olano-Martín y col., 2001), isomaltooligosacáridos (Zhang y col., 2010), fructanos (Moerman y col., 2004), etc., como para reducir el contenido de mono- y disacáridos en mezclas complejas de oligosacáridos, como la lactosa de una fracción de carbohidratos de leche de vaca (Mehra y col., 2014). Goulas y col. (2003) compararon distintas membranas de nanofiltración en modo de diafiltración discontinua para purificar los oligosacáridos derivados de panosa. Sin embargo, en todos estos procesos los resultados de recuperación y pureza de los oligosacáridos dependerán en gran medida del tipo de muestra, de la membrana a utilizar, de las concentraciones de los carbohidratos presentes en la muestra y de las condiciones en la que se lleve a cabo la ultra- o nanofiltración (Hernandez y Ruiz-Matute, 2009).

Por otro lado, la **SEC** es una técnica de separación ampliamente usada para el fraccionamiento de los carbohidratos en función de su grado de polimerización (DP). Se basa en el empleo de columnas rellenas de un gel poroso, donde los carbohidratos se separan de acuerdo con su peso molecular y por la relación entre sus dimensiones moleculares y el diámetro medio de los poros de la fase estacionaria. A modo de ejemplo, se ha empleado para la purificación de GOS comerciales para posteriormente estudiar sus actividades anti-adherentes y prebióticas (Shoaf y col. 2006, Huebner y col. 2007). Esta técnica permite la obtención de altos rendimientos y purezas aceptables, aunque es un proceso largo y tedioso (Hernández y col. 2009).

Otra de las técnicas de fraccionamiento utilizadas para los carbohidratos es la cromatografía **de adsorción en carbón activo**. El uso de diferentes concentraciones de etanol permite una extracción selectiva de los carbohidratos, previamente adsorbidos en el carbón, en función de su grado de polimerización. Además del mecanismo de adsorción de los carbohidratos sobre la matriz de carbón, la retención se produce también por interacciones hidrofóbicas y de intercambio de electrones (Koizumi, 2002). Se ha utilizado ampliamente para la eliminación de monosacáridos en mieles, con objeto de analizar carbohidratos minoritarios de estas muestras (Morales y col., 2006; Ruiz-Matute y col. 2008) y para la eliminación de mono- y disacáridos de una mezcla de GOS con un DP de hasta 7 unidades, obteniendo fracciones de oligosacáridos (DP 3-7) de alta pureza (Sanz y col. 2007). Recientemente, Robinson y col. (2018) han optimizado un método basado en la adsorción en carbón activo empleando cartuchos de SPE (Solid Phase Extraction), que permitía la eliminación de la lactosa de leche bovina y la concentración de los oligosacáridos. Sin embargo, la separación no era sencilla ya que la lactosa y los oligosacáridos poseen estructuras muy parecidas y por tanto poseen afinidad similar con el carbón activo. Tras introducir acetonitrilo (ACN) y ácido trifluoroacético (TFA) en el agua de lavado, consiguieron incrementar la retención de oligosacáridos ácidos y eliminar la mayor parte de lactosa, sin embargo, las muestras todavía contenían 28 mg L^{-1} de lactosa. Para su completa eliminación fue necesario el empleo de tratamientos adicionales basados en la hidrólisis enzimática de la lactosa (De Moura Bell y col., 2016).

Las resinas de **intercambio iónico** son sólidos sintéticos e insolubles en agua, habitualmente con forma de esfera o perla, aunque también pueden encontrarse en polvo. Se componen de grupos polares, ácidos o básicos, en una alta concentración. Estos están unidos a una matriz de un polímero sintético y trabajan obteniendo iones de las soluciones (normalmente agua) y cediendo otros de manera equivalente. Dependiendo de su aplicación se puede emplear la cromatografía de intercambio catiónico o de intercambio aniónico. Se ha empleado para el fraccionamiento selectivo de carbohidratos con cargas, como los iminoazúcares (Molyneux y col., 2002; Asano y col., 2005; Yan y col., 2013).

Los tratamientos basados en el empleo de **disolventes orgánicos** se siguen empleando habitualmente para al fraccionamiento de carbohidratos. Estos tratamientos se basan principalmente en las diferencias de solubilidad de estos compuestos, dando lugar a la precipitación selectiva de algunos de ellos y permitiendo así ser posteriormente separados de la mezcla en la que están presentes. Así, por ejemplo, la tagatosa y la lactulosa, obtenidas por reacciones de isomerización o tratamientos biológicos a partir de sus aldosas (galactosa o lactosa), presentan una mayor solubilidad que éstas en alcoholes como el metanol o el etanol (Montañés y col. 2007) y mezclas hidroalcohólicas (Montañés y col. 2007). Sin embargo, la separación utilizando estos disolventes requiere grandes volúmenes y largos tiempos de tratamiento. Por ello, para minimizar los volúmenes de disolventes usados y los tiempos requeridos en el procedimiento, se recurre a técnicas asistidas ya sea por microondas, líquidos presurizados (PLE; Ruiz-Matute y col., 2007) o usando fluidos supercríticos (SFE; Montañés y col., 2006; Montañés y col., 2009). La PLE también se ha usado en combinación con carbón activo para la obtención de fracciones ricas en oligosacáridos en miel con altos valores de recuperación (Ruiz-Matute y col., 2008). Estas técnicas poseen ventajas en cuanto a la mayor rapidez, reducción del consumo de disolventes y mayor eficacia de fraccionamiento. Sin embargo, su uso para este fin todavía está poco extendido, aplicándose más para la extracción de carbohidratos (Martínez-Villaluenga y col. 2004; Fuentes-Alventosa y col. 2009).

Asimismo, los **tratamientos microbiológicos,** tales como los basados en el empleo de enzimas o levaduras, pueden ser empleados como métodos de fraccionamiento o de eliminación de carbohidratos interferentes. Es conocido que levaduras como *Saccharomyces cerevisiae* presentan una alta especificidad para eliminar algunos mono- y disacáridos presentes en mezclas complejas de carbohidratos (Yoon y col., 2003). Por tanto, las levaduras se han empleado en diversos estudios para eliminar algunos carbohidratos de las muestras de interés. Así por ejemplo, Ruiz-Aceituno y col. (2013) usaron la levadura *Saccharomyces cerevisiae* para la eliminación de los azúcares presentes en legumbres y obtener así extractos ricos en inositoles. Esta misma levadura ha sido empleada por Sanz y col. (2005) para la obtención de una fracción de oligosacáridos de miel libre de glucosa y fructosa y por Ruiz-Matute y col. (2007) para el análisis de compuestos minoritarios en mieles, detectándose dianhídridos de fructosa en mieles adulteradas con distintos jarabes comerciales. Sin embargo, durante el tratamiento con levaduras se producen otros subproductos tales como trehalosa, manitol, glicerol y etanol.

El uso de enzimas también puede ser un método eficaz para el fraccionamiento de carbohidratos, aunque no ha sido muy empleado hasta el momento. Se han usado enzimas de origen fúngico y bacteriano para la eliminación de carbohidratos interferentes. Por ejemplo, Splechtna y col. (2001) emplearon enzimas procedentes de *Sulfolobus solfataricus* and *Pyrococcus furiosus* que producían la ruptura del enlace glicosídico de la lactosa, más selectivamente que el de los GOS, permitiendo la eliminación de este disacárido y dando lugar a glucosa y galactosa. Sin embargo, durante dichas reacciones, se pueden obtener mezclas de distintos oligosacáridos mediante transgalactosilación enzimática con distintas estructuras (longitud de cadena, composición en monosacáridos, grado de ramificación), modificándose la composición original de la muestra de partida. Crittenden y Playne (2002) describieron el uso de *Zymomonas mobilis* para eliminar glucosa, fructosa y sacarosa de inulin-, fructo-, malto-, isomalto- y gentio-oligosacáridos mediante la fermentación de estos carbohidratos pequeños en etanol y dióxido de carbono.

Hasta el momento los datos existentes en la bibliografía sobre el empleo de lactasas (e.g.: Maxilact (DSM, Holanda) o HA-lactase (Christian-Hansen, Dinamarca), se centran en la elaboración de alimentos sin lactosa para el consumo de intolerantes a este carbohidrato (Torres 2012). Se tratan de *β*-D-galactosidasas producidas, principalmente, por *Kluyveromyces lactis* y su uso es de especial interés ya que reducen problemas de salud de personas intolerantes a la lactosa al aumentar su digestibilidad (Jurado, 2004; Ricardi, 2017).

En 2017, Aquino y col. purificaron oligosacáridos de leche de cabra (COS) para aplicarlo a escala industrial, eliminando la lactosa mediante una β-galactosidasa de *Aspergillus oryzae* para facilitar la posterior nanofiltración que permitiera concentrar los COS. Sin embargo, en este estudio no se evalúa la posible degradación de los COS durante el proceso ni la formación de nuevos oligosacáridos por transgalactosilación, que daría lugar a nuevos oligosacáridos no presentes en la leche de cabra. Hasta el momento, no se conocen estudios sobre el análisis de COS tratados con estas enzimas para la eliminación de la lactosa.

En un estudio realizado por Hernández y col. (2009) se compararon cuatro técnicas diferentes de fraccionamiento (diafiltración, tratamiento con levaduras, carbón activo y SEC) para el fraccionamiento de GOS prebióticos comerciales que poseían altas concentraciones de carbohidratos digeribles no deseados (mono- y disacáridos). El empleo de levaduras o carbón activo fue más efectivo para eliminar monosacáridos de las muestras comerciales, en cambio la SEC proporcionó mejores resultados de pureza y rendimiento, facilitando además la purificación de GOS con diferentes DP.

1.3.2. Caracterización de carbohidratos

El análisis de oligosacáridos supone un gran reto para la investigación, principalmente debido a la similitud estructural de estos compuestos, a la ausencia de grupos fluorescentes o cromóforos en sus estructuras y a su falta de volatilidad. Por otra parte, estos oligosacáridos generalmente se encuentran formando mezclas complejas de distintos grados de polimerización, composición monomérica y enlaces glicosídicos. Hay que tener en cuenta también la dificultad que supone la presencia de carbohidratos de menor peso molecular en mayores concentraciones, principalmente monosacáridos y algún disacárido, que dificultan el análisis de carbohidratos minoritarios. El hecho de que la disponibilidad comercial de patrones sea limitada, dificulta aún más su caracterización.

En la bibliografía se han descrito distintos métodos de caracterización de carbohidratos, tales como métodos químicos, que dan lugar a compuestos coloreados y se determinan por gravimetría, espectrofotometría o valoraciones colorimétricas (Brummer y col., 2005) y ensayos microbiológicos o enzimáticos (Anderson 1972). Son métodos sencillos y relativamente rápidos, sin embargo, únicamente proporcionan una medida global del contenido en carbohidratos de una muestra y su exactitud y precisión es limitada. Son, en cambio, las técnicas cromatográficas (Sanz y col., 2009) y electroforéticas (Kong y col., 2008) las más empleadas para el análisis de mezclas complejas de carbohidratos, debido a las ventajas que presentan como su alta resolución, selectividad y sensibilidad, entre otras. De entre ellas, destaca el empleo de la cromatografía de gases (GC) y la cromatografía de líquidos (LC).

1.3.2.1 Cromatografía de gases

La GC es la técnica más utilizada para el análisis de carbohidratos de bajo peso molecular por su alto poder de resolución y elevada sensibilidad proporcionada por los detectores a los que se acopla. Sin embargo, previo a su análisis es necesaria una etapa de **derivatización,** ya que estos compuestos, debido a la presencia de múltiples grupos polares (-OH) en su estructura, presentan baja volatilidad y estabilidad térmica. De este modo, se transforman en sus derivados volátiles, reemplazando los hidrógenos activos por otros grupos apolares, normalmente mediante alquilación, acetilación o sililación:

- Los **acetil derivados** dan lugar a la formación de múltiples picos cromatográficos para un mismo compuesto, debido al efecto de mutarrotación. Con el fin de reducir este número es común llevar a cabo la reducción del grupo carbonilo, de modo que las aldosas y las cetosas se transforman en sus correspondientes alditoles, que tras la acetilación dan lugar a los acetatos de alditol, compuestos muy estables. De este modo se elimina el centro anomérico y se obtiene solo un pico para cada azúcar. Sin embargo, con este procedimiento, carbohidratos distintos pueden dar lugar a un mismo alditol, como es el caso de la obtención de manitol al reducir tanto manosa como fructosa, siendo ésta una importante desventaja de estos derivados (Bonaduce 2007).

$$\text{R-OH} \xrightarrow[\text{Piridina}]{(\text{CH}_3\text{-CO})_2} \text{R-O-COCH}_3$$

- Los **metil éteres** fueron los primeros derivados de carbohidratos empleados para el análisis por GC. El tratamiento con yoduro de metilo o reactivos similares transforman los grupos hidroxilo en grupos –OCH_3. En la actualidad, no se utilizan mucho debido a su laboriosa preparación y a la falta de buena resolución en GC.

$$\text{R-OH} \xrightarrow{CH_3\text{-X}} \text{R-O-}CH_3 + \text{HX}$$

- Uno de los métodos más empleados de derivatización es la **sililación**, donde tiene lugar la introducción de grupos dimetilsilil, trimetilsilil (TMS) o *t*-butildimetilsilil en la molécula para formar silil éteres (Chrums 1990). De todos ellos, los más comunes son los TMS derivados por su buena estabilidad y alta volatilidad. Existen diferentes reactivos (trimetilsililimidazol (TMSI); trimetilclorosilano (TMCS); hexametildisilazano (HMDS); *N*-metil-*N*-(trimetilsilil)trifluoroacetamida (MSTFA); *N,O*-bis-(trimetilsilil)-trifluoroacetamida (BSTFA); etc) y condiciones de sililación (tiempos y temperaturas) empleadas que afectan a la reacción (Sanz y Martínez-Castro 2007). Esta técnica de derivatización está muy establecida para carbohidratos neutros, sin embargo, carbohidratos constituidos por grupos ácidos u otros grupos funcionales no se ven tan favorecidos, generando problemas en su análisis y siendo necesaria la optimización del proceso de derivatización en cada caso. Además, con estos derivados se pueden obtener hasta 5 compuestos por cada carbohidrato reductor (α y β furanosa, α y β piranosa y cadena abierta), lo que puede dificultar el análisis de mezclas complejas. Una solución a este problema es la conversión previa a la sililación del grupo carbonilo en una **oxima**, obteniéndose finalmente trimetilsilil oximas (TMSO), lo que reduce a 2 (formas *E* y *Z*) el número de picos cromatográficos y facilita su identificación (Ruiz-Matute y col., 2011) (Figura 1.3). Los azúcares no reductores no experimentan el proceso de oximación y dan lugar a un solo derivado.

$$\text{R-OH} + \text{R'}_3\text{Si-X} \longrightarrow \text{R-O-SiR'}_3 + \text{HX}$$

1. Formación de la oxima 2. Formación del derivado trimetilsililado

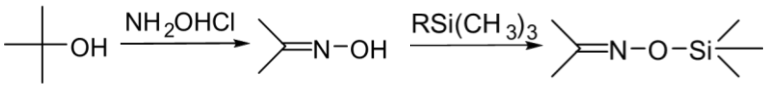

Figura 1.3: Esquema de reacción de la derivatización de un carbohidrato por oximación y trimetilsililación

La GC normalmente se emplea para el análisis de carbohidratos de bajo peso molecular (DP ≤ 3), mediante **columnas** capilares con fases estacionarias de polisiloxano con grupos metil- o fenil-. Estas columnas poseen dimensiones que oscilan entre 10-50 m de longitud, 0,1-0,5 mm de diámetro interno y 0,25-0,5 µm de fase estacionaria y un rango de temperaturas de operación entre 60 y 330 °C. Sin embargo, en la actualidad existe disponibilidad de columnas comerciales que soportan altas temperaturas, como las columnas con grupos carborano presentes en la estructura principal del polisiloxano, fabricadas en capilares de sílice fundida recubiertas de poliimida o de aluminio, y que han permitido extender su aplicación hasta carbohidratos con DP 7 o incluso superior (Sabater y col., 2016).

El **detector** más usado en GC es el de ionización de llama (**FID**), que posee una alta sensibilidad. Sin embargo, en este caso el empleo de patrones para la identificación de carbohidratos es imprescindible. No obstante, su acoplamiento con la espectrometría de masas (**GC-MS**) resulta ventajoso, ya que proporciona información estructural sobre los compuestos analizados, facilitando su identificación. Esta técnica está basada en la producción de iones que posteriormente son separados o filtrados según su relación masa/carga *(m/z)* y finalmente detectados para dar lugar al espectro de masas.

Dependiendo de la fuente de ionización utilizada, se consigue mayor o menor fragmentación de la molécula, pudiéndose determinar el peso molecular del compuesto cuando la ionización es suave, ionización química (CI), o proporcionando información sobre la estructura cuando la ionización es por impacto electrónico (EI). La información estructural proporcionada por el sistema también depende del analizador empleado, permitiendo en algunos casos, como en el de las trampas iónicas (IT) o los triples cuadrupolos (QqQ), la obtención de fragmentos sucesivos a partir de una misma molécula (MS^n o MS^2, respectivamente). Sin embargo, en el caso de los carbohidratos, debido a su similitud estructural, se obtienen patrones de fragmentación con características muy similares para los distintos compuestos, haciendo que su caracterización sea compleja. Un estudio pormenorizado de la relación entre el patrón de fragmentación en espectrometría de masas y la estructura química del carbohidrato puede permitir un avance en la caracterización de los compuestos desconocidos. Tal es el caso de los escasos estudios que se han llevado a cabo hasta el momento para el análisis de TMSO de mono-, di- y trisacáridos (Sanz y col., 2002; Brokl et al., 2009).

1.3.2.2. Cromatografía de líquidos

La cromatografía de líquidos (LC) es la técnica más usada para la caracterización de carbohidratos y en especial de oligosacáridos prebióticos, por su alta versatilidad y múltiples modos de operación. Al contrario que en GC, no es requisito imprescindible para el análisis de carbohidratos por LC llevar a cabo un proceso de derivatización previo; sin embargo, en algunos casos es recomendable o incluso necesario, como se comentará más adelante.

Los modos de operación más empleados para el análisis de oligosacáridos son:

La **cromatografía en fase inversa (RPLC)** emplea fases estacionarias no polares, típicamente de partículas de sílice químicamente modificadas con hidrocarburos de cadena larga, siendo las más comunes las modificadas con cadenas de 18 átomos de carbono (columna C_{18}). En este tipo de cromatografía el mecanismo de retención consiste en interacciones hidrofóbicas de los analitos con la fase estacionaria de la columna. La fase móvil es más polar que la estacionaria y, normalmente, se suelen emplear mezclas de agua y acetonitrilo con modificadores orgánicos como trifluoroacético o fórmico, aumentando la concentración de acetonitrilo a medida que avanza el gradiente. En el caso de carbohidratos, el agua es el eluyente más usado, pero la aplicabilidad de este modo de operación es muy limitada debido a la poca retención de los compuestos (Brokl y col., 2011). En este caso, el empleo de reacciones de derivatización que mejoren las propiedades cromatográficas resulta relevante para el empleo de este modo de operación (Higashi y col., 1990).

La **cromatografía de carbón grafitizado (GCC)** se desarrolló como alternativa a la RPLC. Su mecanismo de separación no es del todo conocido, aunque se sabe que tienen lugar diversas interacciones, tales como repulsiones hidrofóbicas entre eluyente y analito, interacciones de los grupos funcionales polarizados del analito con el grafito, etc. Una de las ventajas de este tipo de cromatografía es su capacidad de separar isómeros en presencia de modificadores básicos, como el hidróxido de amonio, así como la estabilidad de la fase estacionaria en todo el rango de pH y el uso de fases móviles totalmente compatibles con MS (Koizumi, 2002). En general, la mayoría de las aplicaciones se orientan al análisis de glicanos y glicopéptidos (Ruhaak y col., 2009). Recientemente, se han empleado columnas de carbón grafitizado para la separación de carbohidratos prebióticos, resultando ser adecuadas para la separación de isómeros, aunque se observaron coeluciones entre oligosacáridos de distinto grado de polimerización, causando problemas en el análisis de mezclas muy complejas (Brokl y col., 2011).

La **cromatografía de alta eficacia de intercambio aniónico (HPAEC)** es una de las técnicas más difundidas para la caracterización de carbohidratos (Coulier y col., 2009; Borromei y col., 2010; Avila-Fernandez y col., 2011; Blanch y col., 2011). Este tipo de cromatografía se lleva a cabo a elevados valores de pH, a los que los carbohidratos son ionizados para ser separados en resinas de intercambio aniónico. La detección se lleva a cabo utilizando amperometría de pulsos (PAD), que consiste en la medida de la corriente eléctrica generada por la oxidación de los carbohidratos en la superficie de un electrodo de platino u oro y su posterior reducción para limpieza del electrodo. Sin embargo, esta técnica presenta dos grandes desventajas: (i) los imprevisibles comportamientos de elución relacionados con la estructura de cada carbohidrato, lo cual hace imprescindible el uso de patrones para la identificación de cada compuesto y (ii) su difícil acoplamiento a la espectrometría de masas debido a la presencia de altos contenidos de sales, como el acetato de sodio, usado como modificador en la fase móvil, o de altos contenidos de hidróxido de sodio. Recientemente, se están desarrollando nuevos sistemas que facilitan estos acoplamientos creando sistemas más robustos (Maier y col., 2016; Coulier, 2013).

La **cromatografía de alta eficacia de intercambio catiónico (HPCEC)** separa los iones y moléculas polares basándose en su carga. La fase estacionaria contiene grupos funcionales cargados negativamente a los que están unidos los denominados contraiones con los que compiten en interacción electrostática los compuestos de interés. Las típicas fases estacionarias utilizadas para el análisis de oligosacáridos son las de poliestireno sulfonado con contraiones Ca^{2+} y Ag^{2+}, mientras que para los monosacáridos se emplean las de Pb^{2+} (Verzele 1987).

En los últimos años, uno de los modos de operación más utilizados para el análisis de carbohidratos por LC es la **cromatografía de interacción hidrofílica (HILIC).** La HILIC se presenta frecuentemente como una variante de la cromatografía de fase normal (NPLC), puesto que al igual que ésta, utiliza una fase estacionaria polar y la retención de los analitos aumenta con el incremento de su hidrofilia, aunque su mecanismo es mucho más complejo y, todavía, genera controversia entre la comunidad científica (Greco y Letzel, 2013). Esta técnica resulta de gran utilidad para analizar compuestos que, dada su elevada polaridad, se retienen poco en RPLC y eluyen cerca del frente del disolvente, como ocurre con los carbohidratos. Estos compuestos además muestran una buena solubilidad en la fase móvil acuosa utilizada en HILIC, lo que contribuye a solventar los frecuentes problemas relacionados con la baja solubilidad de estos compuestos en las fases de NPLC. Aunque cualquier fase estacionaria que pueda retener moléculas de agua, puede ser usada en modo HILIC (Greco y Letzel, 2013), la fase estacionaria típica consiste en la clásica gel de sílice modificada con distintos grupos funcionales polares (amino, diol, amida, etc). En esta sección se ha considerado de interés incluir como anexo un artículo de revisión, titulado “Análisis de carbohidratos bioactivos mediante cromatografía de interacción hidrofílica (HILIC)” de M.J. García-Sarrió, S. Rodríguez-Sánchez y **A. Martín-Ortiz** publicado en la revista Cromatografía y Técnicas Afines 35 (2) 2014, 47-59 (**Anexo 1.1.**), que revisa el empleo de la HILIC para el análisis de carbohidratos bioactivos. En este anexo se comentan los mecanismos, fases estacionarias, condiciones cromatográficas y aplicaciones de HILIC para el análisis de estos compuestos.

En general, el análisis de carbohidratos por LC se lleva a cabo empleando columnas convencionales, de 10 - 15 cm de longitud, 2,1 - 4,6 mm de diámetro interno y con partículas 1,7 - 5 µm (Ruhaak y col., 2009; Marrubini y col., 2018). En la actualidad hay una tendencia a la miniaturización, tanto del diámetro interno de las columnas, como de la longitud, dando lugar al uso de nano-chips (Figura 1.4), que resulta de gran interés ya que su empleo se traduce en un menor tiempo de análisis y se requiere el empleo de menor cantidad de muestra (Zotou, 2012; Cohen y col., 2017). Esta cromatografía se ha desarrollado con éxito para el análisis de oligosacáridos de leche humana (Niñonuevo y col. 2008, Wu y col. 2011), leche de cerdos (Tao y col. 2010) y leche de vaca (Tao y col. 2008, Barile y col. 2010), etc. En la cromatografía líquida de ultra alta resolución (UPLC) se emplean columnas con tamaño inferior a 2 µm, de este modo se reduce el tiempo de análisis y se mejora la forma de los picos (Jiao y col., 2017).

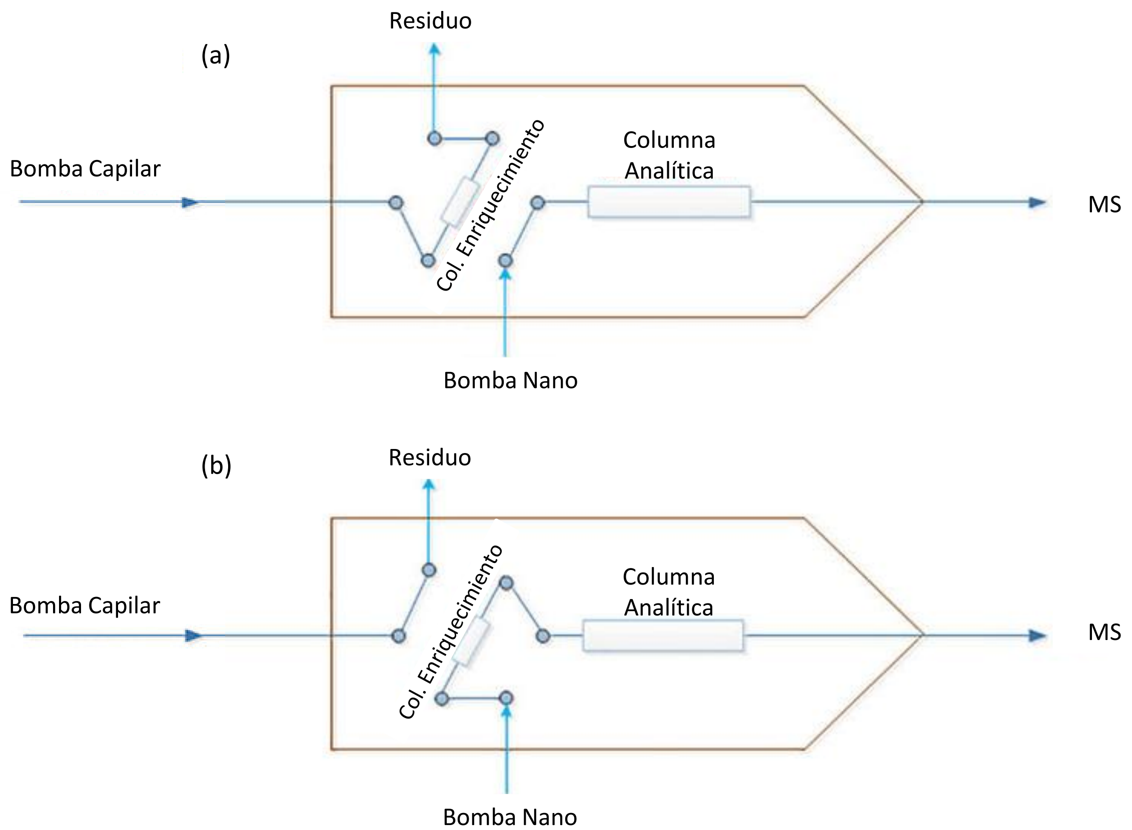

Figura 1.4: Esquema de un nano-chip. (a) Los compuestos son retenidos en la columna de enriquecimiento. (b) Cambio de posición y los analitos previamente retenidos son eluidos y analizados en la columna analítica.

Por otra parte, debido a la falta de grupos cromóforos, la **detección** de carbohidratos por LC resulta difícil. Entre los detectores más comúnmente empleados en LC, destaca el de índice de refracción (RI) (aunque sólo se puede utilizar cuando el modo de elución es isocrático), y el detector amperométrico de pulsos (PAD). Éste último ofrece un sistema de detección sensible (límite de detección entre 0,01 y 1 ng) y de alta selectividad, no requiere de grupos cromóforos o fluorescentes, por lo que ha sido muy utilizado en el análisis de oligosacáridos (Morales y col., 2008; Borromei y col., 2010)

Los detectores de ultravioleta (UV) o fluorescencia se pueden emplear también, pero, dada la ausencia de grupos cromóforos o fluoróforos en los carbohidratos, es necesario llevar a cabo una reacción de derivatización previa a la detección de los compuestos. Existen varios métodos de derivatización que introducen antes, durante o después de la separación grupos cromóforos en la molécula para su detección por UV o fluoróforos para la detección fluorescente (Lamari, 2003). La mayoría de los métodos se basan en la condensación del grupo carbonilo de los carbohidratos con aminas primarias, para dar lugar a una base de Schiff, que es posteriormente reducida a una glicosil-amina *N*-sustituída (Hase, 1996). Esta amina debe de poseer el grupo cromóforo o fluoróforo deseado (generalmente un anillo aromático) (Sanz y Martínez-Castro, 2007). La reducción se puede llevar a cabo empleando 2-aminopiridina (2-AP), diferentes trisulfonatos, ésteres del ácido p-amino benzoico, etc. Entre estos reactivos típicamente utilizados, cabe destacar el 4-aminobenzoato de etilo (ABEE) que, en medio ácido y metanol, produce la reducción del grupo amino, convirtiéndolo en un producto que absorbe la luz ultravioleta (Wang y col., 1984, Kwon and Kim, 1993, Yasuno y col., 1997). La figura 1.5 muestra un esquema de esta reacción. Estos carbohidratos derivatizados presentan también una mayor retención en cromatografía de fase inversa, mejorando así la resolución de los mismos (Higashi y col., 1990).

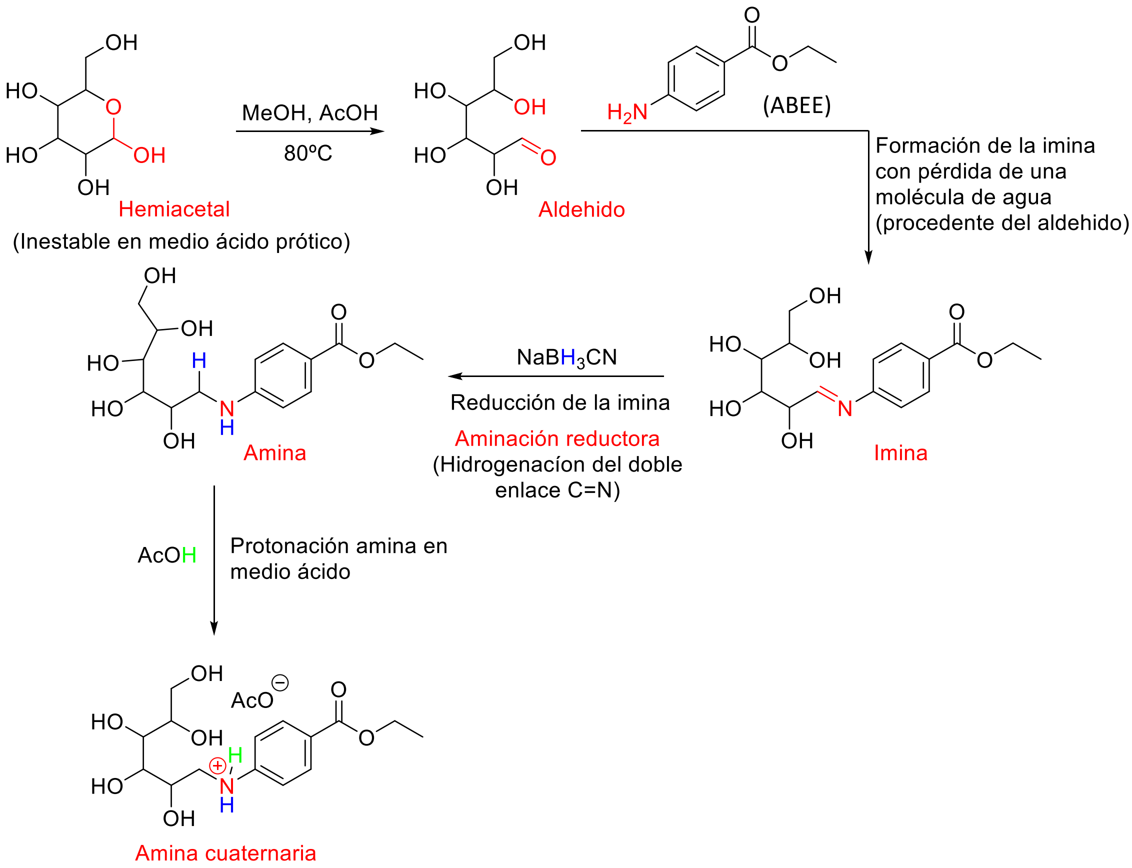

Figura 1.5: Esquema de reacción de derivatización de un carbohidrato con el reactivo ABEE en el medio de reacción metanol, ácido acético y cianoborohidruro de sodio.

Al igual que en el caso de la GC, el acoplamiento de **espectrómetros de masas** a equipos de LC proporciona grandes ventajas para el análisis de carbohidratos, ya que aporta información adicional sobre su estructura. Uno de los requisitos principales para llevar a cabo dicho acoplamiento es el uso de disolventes volátiles y agua, adecuados para ser introducidos en el espectrómetro. La fuente de ionización más común para el análisis de carbohidratos, es la electronebulización o ionización por electroespray (ESI), aunque también se emplea la ionización química a presión atmosférica (APCI). Los analizadores empleados son muy numerosos, desde cuadrupolos simples (Q), hasta IT, analizadores de tiempo de vuelo (ToF), y variantes de los mismos como QqQ o cuadrupolo acoplado a tiempo de vuelo (Q-ToF), entre otros.

Mientras que los analizadores de Q o ToF únicamente proporcionan información sobre el grado de polimerización del oligosacárido (ión cuasimolecular), el empleo de analizadores que permitan aplicar la espectrometría de masas MS/MS o tándem MS como el Q-ToF, QqQ o IT representan una alternativa ventajosa en el análisis estructural de oligosacáridos bioactivos. Así, en estos analizadores se produce la fragmentación de un ión precursor, en iones más pequeños, acompañada por la pérdida de un fragmento neutro. Generalmente, los iones precursores y productos son separados en el tiempo si se realizan diferentes etapas secuenciales en el mismo analizador (IT) o separados en el espacio usando analizadores conectados en línea (QqQ y Q-ToF) (Wu y col. 2011). En el caso de la trampa iónica existe una ventaja adicional debido a la capacidad de este analizador para realizar MS^n, lo que permite determinar los enlaces glicosídicos que componen los oligosacáridos. Sin embargo, esta no es una tarea trivial. Son pocos los estudios realizados hasta el momento en los que se determinan las estructuras químicas de oligosacáridos prebióticos mediante MS^n (Hernández-Hernánez et al., 2012), siendo de especial interés profundizar en este campo.

1.3.2.3. Técnicas multidimensionales

En muchos casos las técnicas cromatográficas clásicas, incluso de alta eficacia, no son capaces de resolver completamente algunas de las complejas mezclas de carbohidratos presentes en los alimentos o en los ingredientes alimentarios. Un aumento importante del poder de separación se consigue mediante el acoplamiento de dos técnicas cromatográficas, lo que se denomina cromatografía multidimensional o en dos dimensiones.

En la cromatografía multidimensional clásica (GC-GC, LC-LC,…), dos columnas de dimensiones convencionales con fases estacionarias distintas se acoplan en serie mediante una interfase, que permite la transferencia selectiva de una o varias fracciones (heart-cuts) de la primera a la segunda columna. En cambio, en las técnicas cromatográficas completas en dos dimensiones o bidimensionales (LC×LC, GC×GC) todo el eluyente de la primera columna es transferido a la segunda dimensión en la que la separación tiene lugar en condiciones de cromatografía rápida. Así, la totalidad de la muestra es sometida a dos procesos de separación diferentes en un único análisis (el tiempo de análisis total es similar al de un análisis monodimensional), pero siempre preservando la separación conseguida en la primera dimensión, consiguiendo así un aumento importante del poder de separación (Montero y col., 2017).

Hasta el momento únicamente la cromatografía de gases completa en dos dimensiones (GC×GC) se ha aplicado al análisis de carbohidratos (Marsol-Vall y col., 2015, Brokl y col., 2010). En **GC×GC**, dos columnas con fases estacionarias de distinta polaridad se acoplan mediante una interfase o modulador. La combinación de columnas más empleadas es una no polar en la primera dimensión (^{1}D) y otra polar en la segunda (^{2}D). Es la denominada combinación normal, separándose los analitos en ^{1}D en función de su diferente volatilidad, mientras que en ^{2}D lo hacen por sus interacciones específicas con la fase estacionaria, dando lugar a una separación pseudo-ortogonal. El principal elemento de esta técnica es el **modulador**, que es el que permite preservar la separación de los analitos conseguida en la ^{1}D en el análisis en la ^{2}D. Existen varios tipos de moduladores siendo los más utilizados los térmicos y en concreto, los criogénicos. En estos moduladores se atrapan los analitos que eluyen de la ^{1}D mediante nitrógeno líquido o anhídrido carbónico criogénico para su posterior volatilización y reinyección como banda enfocada en la ^{2}D mediante aire caliente (Mondello y col. 2008).

La GC×GC se ha empleado en el análisis de carbohidratos de la miel, consiguiendo separar la mayoría de los disacáridos de la miel por primera vez (Brokl y col., 2010) o en el análisis de disacáridos de manzana y melocotón (Marsol-Vall y col., 2015) identificando hasta 10 disacáridos. También se ha aplicado con éxito al análisis de enantiómeros de monosacáridos (Myrgorodska, 2017).

En el caso de cromatografía de líquidos bidimensional (LC×LC), hasta el momento no existen estudios relativos al análisis de carbohidratos debido a la dificultad que conlleva encontrar combinaciones de columnas que tengan buena ortogonalidad, o a desajustes causados por los diferentes disolventes de cada dimensión o las complicaciones para detectar los carbohidratos, siendo ésta, por tanto, una línea de investigación futura.

1.4. Bibliografía

Anderson, L., (1972) The cyclitols. En: The carbohydrates, chemistry and biochemistry. W Pigman, D Horton. New York-London, Academic Press, 519-579.

Aquino, L., de Moura Bell, J., Cohen, J.L., Liu, Y., Lee, H., de Melo Silva, V.L., Domizio, P., Conte Junior, C.A., Barile, D., (2017) Purification of caprine oligosaccharides at pilot-scale. Journal of Food Engineering, 214, 226-235.

Asano N., Yamauchi T., Kagamifuchi K., Shimizu N., Takahashi S., Takatsuka H., Ikeda K., Kizu H., Chuakul W., Kettawan A., Okamoto T., (2005) Iminosugar-producing Thai medicinal plants. Journal of Natural Products, 68, 1238-1242.

Avila-Fernandez, A., Galicia-Lagunas, N., Rodriguez-Alegria, M.E., Olvera, C., y Lopez-Munguia, A., (2011) Production of functional oligosaccharides through limited acid hydrolysis of agave fructans. Food Chemistry, 129, 380-386.

Barile, D., Marotta, M., Chu, C., Mehra, R., Grimm, R., Lebrilla, C.B., German, J.B., (2010) Neutral and acidic oligosaccharides in Holstein–Friesian colostrum during thefirst 3 days of lactation measured by high performance liquid chromatographyon a microfluidic chip and time-of-flight mass spectrometry, Journal of Dairy Science, 93, 3940-3949.

Barile, D., Rastall, R. A., (2013) Human milk and related oligosaccharides as prebiotics. Current Opinion in Biotechnology, 24 (2), 214-219.

Belitz, H.D., Grosch, W., Schieberle, P., (2009) Carbohydrates, Food Chemistry. HD Belitz, W Grosch,P Schieberle. Berlin, Springer-Verlag Berlin Heidelberg, 248-339.

Blanch, M., Sanchez-Ballesta, M.T., Escribano, M.I., y Merodio, C., (2011) Fructooligosaccharides in table grapes and response to storage. Food Chemistry, 129, 724-730.

Bode, L., (2006) Recent advances on structure, metabolism, and function of human milk oligosaccharides. Journal of Nutrition, 136 (8), 2127-2130

Bode, L., (2012) Human milk oligosaccharides: Every baby needs a sugar mama. Glycobiology, 22 (9), 1147-1162.

Boehm, G., Stahl, B., (2007). Oligosaccharides from milk. The Journal of Nutrition, 137, 847-849.

Bonaduce, I., Brecoulaki, H., Colombini, M., Lluveras, A., Restivo, V., Ribechini, E., (2007) Gas chromatographic-mass spectrometric characterisation of plant gums in samples from painted Works of art. Journal of Chromatography A, 1175, 275.

Borromei, C., Cavazza, A., Corradini, C., Vatteroni, C., Bazzini, A., Ferrari, R., y Merusi, P., (2010) Validated HPAEC-PAD method for prebiotics determination in synbiotic fermented milks during shelf life. Analytical and Bioanalytical Chemistry, 397, 127-135.

Brokl, M., Soria, A.C., Martínez-Castro, I., Sanz, M.L., Ruiz-Matute, A.I., (2009) Characterization of O-trimethylsilyl oximes of trisaccharides by gas chromatography–mass spectrometry. Journal of Chromatography A, 1216 (46) 89-4692.

Brokl, M., Soria, A.C., Ruiz-Matute, A.I., Sanz, M.L., Ramos, L., (2010) Separation of disaccharides by comprehensive two-dimensional gas chromatography−time-of-flight mass spectrometry. Application to honey analysis. Journal of Agricultural and Food Chemistry, 58, 11561–11567.

Brokl, M., Hernández-Hernández, O., Soria, A.C., y Sanz, M.L., (2011) Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. Journal of Chromatography A., 1218, 7697-7703.

Brummer, Y. y Cui, S.W., (2005) Understanding carbohydrates analysis, en Food Carbohydrates. Chemistry, physical properties and applications, Editor S.W. Cui, Taylor & Francis Group: Boca Raton.

Chichlowski, M., German, J.B., Lebrilla, C.B., Mills, D.A., (2011). The influence of milk oligosaccharides on microbiota of infants: opportunities for formulas. Annual Review in Food Science and Technology, 2, 1-21.

Chrums, S.C., (1990) Recent developments in the chromatographic analysis of carbohydrates“. Journal of Chromatography A, 500, 555.

Chung, M. S., Ruan, R. R., Chen, P. L., & Wang, X., (1999) Physical and chemical properties of caramel systems. Food Science and Technology – Lebensmittel-Wissenschaft & Technologie, 32(3), 162–166.

Claude, J., Ubbink, J., (2006). Thermal degradation of carbohydrate polymers in amorphous states: A physical study including colorimetry. Food Chemistry 96 (3), 402-410.

Cohen, J.L., Barile, D.,Liu, Y., de Moura Bella, J.M.L.N., (2017) Role of pH in the recovery of bovine milk oligosaccharides from colostrum whey permeate by nanofiltration. International Dairy Journal, (66) 68-75.

Coppa, G., Bruni, S., Morelli, L., Soldi, S., Gabrielli, O., (2004) The first prebiotics in humans - Human milk oligosaccharides. Journal of Clinical Gastroenterology, 38, S80-S83.

Corzo, N., Alonso, J. L., Azpiroz, F., Calvo, M. A., Cirici, M., Leis, R., Lombó, F., Mateos-Aparicio, I., Plou, F. J., Ruas-Madiedo, P., Rúperez, P., Redondo-Cuenca, A., Sanz, M. L., Clemente, A., (2015) Prebióticos; concepto, propiedades y efectos beneficiosos Ref. Revista/libro: Nutrición Hospitalaria, 31 (1), 99-121.

Coulier, L., Timmermans, J., Bas, R., Van Den Dool, R., Haaksman, I., Klarenbeek, B., Slaghek, T., y Van Dongen, W., (2009) In-Depth Characterization of Prebiotic Galacto-oligosaccharides by a Combination of Analytical Techniques. Journal of Agricultural and Food Chemistry, 57, 8488-8495.

Coulier, L., Zha, Y., Bas, R., Punt, P.J., (2013) Analysis of oligosaccharides in lignocellulosic biomass hydrolysates by high-performance anion-exchange chromatography coupled with mass spectrometry (HPAEC-MS). Bioresource Technology, 133, 221-231.

Crittenden, R.G.; Playne, M.J., (2002) Purification of food-grade oligosaccharides using immobilised cells of Zymomonas mobilis. Applied Microbiology and Biotechnology, 58 (3), 297-302.

De Moura Bell, J. M., Aquino, L. F., Liu, Y., Cohen, J. L., Lee, H., de Melo Silva, V. L., Rodrigues M.I., Barile D., (2016) Modeling lactose hydrolysis for efficiency and selectivity: Toward the preservation of sialyloligosaccharides in bovine colostrum whey permeate. Journal of Dairy Science, 99, 6157-6163.

Fuentes-Alventosa, J., Jaramillo-Carmona, S., Rodríguez-Gutiérrez, G., Rodríguez-Arcos, R., Fernández-Bolaños, J., Guillén-Bejarano, R., Espejo-Calvo, J., Jiménez-Araujo, A., (2009) Effect of the extraction method on phytochemical composition and antioxidant activity of high dietary fibre powders obtained from asparagus by-products. Food Chemistry, 116(2), 484-490.

Gilar, M., Bouvier, E.S.P., y Compton, B.J., (2001) Advances in sample preparation in electromigration, chromatographic and mass spectrometric separation methods. Journal of Chromatography A, 909, 111-135.

Giorgio, D., Di Trana, A., Claps, S., (2018) Oligosaccharides, polyamines and sphingolipids in ruminant milk. Small Ruminant Research, 160, 23-30.

Goulas, C., Zervas, G., Papadopoulos, G., (2003) Effect of dietary animal fat and methionine on dairy ewes milk yield and milk composition. Animal Feed Science and Technology, 105 (1-4), 43-54.

Greco, G., Letzel T., (2013) Main Interactions and Influences of the Chromatographic Parameters in HILIC Separations. Journal of Chromatography and Science, 51, 1-10.

Grindley, T., (2008) Structure and Conformation of Carbohydrates. In: Fraser-Reid B.O., Tatsuta K., Thiem J. (eds) Glycoscience. Springer, Berlin, Heidelberg

Guarner, F., (2009). Prebiotics, porbiotics and helminths: the “natural” solution. Digestive Diseases, 27, 412-417 .

Hase, S., (1996) Precolumn derivatization for chromatographic and electrophoretic analyses of carbohydrates. Journal of Chromatography A, 720 (1-2), 173-182.

Hernández, O., Ruiz-Matute, A.I., Olano, A., Moreno, F.J., Sanz, M.L., (2009). Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. International Dairy Journal, 19 (9), 531-536.

Hernandez, O., Ruiz-Matute, A.I., (2008) Fundamentos y aplicaciones de las técnicas de fraccionamiento de carbohidratos. Cromatografía y Técnicas Afines, 29 (1), 3-14.

Hernández-Hernández, O., Calvillo, I., Lebrón-Aguilar, R., Moreno, F.J., Sanz, M.L., (2011) Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides. Journal of Chromatography A., 1220, 57-67.

Higashi, H., Ito, M.,Fukaya, N., Yamagata, S., Yamagat, T., (1990) Two-dimensional mapping by the high performance liquid chromatography of oligosaccharides released from glycosphingolipids by endoglycoceramidase, Analytical Biochemistry, 186, 355-362.

Huebner, J., Wehling, R., y Hutkins, R. (2007) Functional activity of commercial prebiotics. International Dairy Journal, 17, 770-775.

IUPAC Nomenclature of carbohydrates (IUPAC recommendations 1996). Pure and applied chemistry, 68, 1919.

Jéquier, E. (1994) Carbohydrates as a source of energy. The American Journal of Clinical Nutrition, 59 (3), 1

Jiao, Y., Li, F., Jiang, D., Li, W., Chen, J., (2016) Determination of Isomaltooligosaccharides in Milk Powder by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry. Analytical Letters, 49 (4) 458-466.

Jurado, E., Camacho, F., Luzón, G., Vicaria, J.M., (2004) Kinetic models of activity for β-galactosidases: influence of pH, ionic concentration and temperature. Enzym. Microb. Technol., 34, 33-40.

Koizumi, K., (2002) HPLC of carbohydrates on graphitizaed carbon columns, en Carbohydrate analysis by modern chromatography and elcetrophoresis, Editor Z. El Rassi, Elsevier: Amsterdam. 103-118.

Kong, L., Wang, Y., Cao, Y., (2008) Determination of myo-inositol and d-chiro-inositol in black rice bran by capillary electrophoresis with electrochemical detection. Journal of Food Composition and Analysis, 21(6), 501-504.

Kunz, C., Kuntz, S., Rudloff, S., (2014) Bioactivity of human milk oligosaccharides, Food oligosaccharides: production, analysis and bioactivity. (Ed) Moreno y Sanz. Wiley Blackwell

Kunz, C., Rudloff, S., Baier, W., Klein, N., Strobel, S., (2000). Oligosaccharides in human milk: structural, functional and metabolic aspects. Annual Reviews of Nutrition 20, 699-722.

Kwon, H., Kim, J., (2013) Determination of monosaccharides in glycoproteins by reverse-phase high-performance liquid-chromatography. Analytical biochemistry, 215, 243-252.

Lamari, F.N., Kuhn, R., Karamanos, N.K., (2003) Derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometris structure analysis. Journal of Chromatography B, 793 15

Lane, J.A., Mehra, R.K., Carrington, S.D., Hickey, R.M., (2010) The food glycome: a source of protection against pathogen colonization in the gastrointestinal tract. International Journal of Food Microbiology, 142, 1-13.

Langana, A. S., Garzon, S., Casarin, J. (2018) Inositol in polycystic ovary Syndrome: Restoring Fertility through a Pathophysiology-Based Approach. Trends in endocrinology and metabolism, 29 (11) 768-780.

Lopez-Castejon, L., Bengoechea, C., Espinosa, S., (2019) Characterization of prebiotic emulsions stabilized by inulin and beta-lactoglobulin. Food Hydrocolloids, 87, 382-393.

Maier, M., Reusch, D., Bruggink, C., Bulau, P., Wuhrer, M., Mølhøj, M., (2016) Applying mini-bore HPAEC-MS/MS for the characterization and quantification of Fc N-glycans from heterogeneously glycosylated IgGs. Journal of Chromatography B. 1033-1034.

Marrubini, G., Appelblad, P., Maietta, M., (2018). Hydrophilic interaction chromatography in food matrices analysis: An updated review. Food Chemistry, (257) 53-66.

Marsol-Vall, A., Balcells, M., Eras, J., Canela-Garayoa, R., (2015) Comprehensive Two-dimensional Gas Chromatography Time-of-flight Mass Spectrometry to Assess the Presence of alpha,alpha-Trehalose and Other Disaccharides in Apple and Peach. Phytochemical Analysis, 26 (4), 279-286.

Martínez-Ferez, A.; Rudloff, S.; Guadix, A.; Henkel, C. A.; Pohlentz, G.; Boza, J. J.; Guadix, E. M.; Kunz, C., (2006) Goats' milk as a natural source of lactose-derived OS: isolation by membrane technology. International Dairy Journal, 16, 173-181.

Martínez-Villaluenga, C., Frias, J., Gulewicz, K., Vidal-Valverde, C., (2004) Improved Method To Obtain Pure α-Galactosides from Lupin Seeds. Journal of Agricultural and Food Science, 52 (23), 6920-6922.

Matsumoto, K., Kobayahi, Y., Ueyama, S., Watanabe, T., Tanaka, T., Kan, T., kuroda, a., Sumihara, Y., (1993) Galactooligosaccharides, In T. Nakanuki, Oligosaccharides. Production, properties and applications. Japanese Technology Reviews, 3 (2). New york: Gordon: Gordon and breach science publisher. 90-106.

Mehra, R., Barile, D., Marotta M., Lebrilla, C. B., Chu C., German, J. B., (2014) Novel High-Molecular Weight Fucosylated Milk Oligosaccharides Identified in Dairy Streams. Plos One, 9 (5), 96040.

Meyrand, M.; Dallas, D. C.; Caillat, H.; Bouvier, F.; Martin, P.; Barile, D., (2013) Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize αs1-casein. Small Ruminant Research, 113, 411–420.

Moerman, F. T., Van Leeuwen, M. B., Delcour, J. A. (2004). Enrichment of higher molecular weight fractions in inulin. Journal of Agricultural and Food Chemistry, 52 (12), 3780-3783.

Molyneux R.J., Gardner D.R., James L.F., Colegate S.M., (2002) Polyhydroxy alkaloids: chromatographic analysis, Journal of Chromatography A, 967, 57-74.

Mondello, L., Tranchida, P.Q., Dugo, P., Dugo, G. (2008) Comprehensive two-dimensional gas chromatography-mass spectrometry: A review. Mass Spectrometry Reviews, 27, 101.

Mondello, L., Herrero, M., Kumm, T., Dugo, P., Cortes, H., Dugo, G., (2008) Quantification in comprehensive two-dimensional liquid chromatography, Analytical Chemistry, 80 (14) 5418-5424.

Montanes, F., Fornari, T., Martin-Alvarez, P. J., Corzo, N., Olano, A., Ibanez, E., (2006) Selective recovery of tagatose from mixtures with galactose by direct extraction with supercritical CO2 and different cosolvents. Journal of Agricultural and Food Chemistry, 54 (21), 8340-8345.

Montanes, F., Olano, A., Ibanez, E., Fornari, T. (2007) Modeling solubilities of sugars in alcohols based on original experimental data. Aiche Journal, 53 (9) 2411-2418.

Montero, L., Ibañez, E., Russo, M., Rastrelli, L., Cifuentes, A., Herrero, M., (2017) Focusing and non-focusing modulation strategies for the improvement of on-line two-dimensional hydrophilic interaction chromatography x reversed phase profiling of complex food samples. Analitical Chimica Acta, 985, 202-212.

Morales, V., Sanz, M.L., Olano, A., Corzo, N., (2006) Rapid separation on activated charcoal of high oligosaccharides in honey. Chromatographia, 64 (3-4), 233-238.

Morales, V., Corzo, N., Sanz, M.L., (2008) HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups. Food chemistry 107, 922-928

Moreno, F. J., Carrero-Carralero, C., Hernández-Hernández, O., Sanz, M.L. (2014) Fractionation of food bioactive oligosaccharides. Food oligosaccharides: production, analysis and bioactivity. (Ed) Moreno y Sanz. Wiley Blackwell

Moro, G., Stahl, B., Fanaro, S., Jelinek, J., Boehm, G., Coppa, G.V., (2005) Dietary prebiotics oligosaccharides are detectable in the faeces of formula-fed infants. Acta Pediatrae, 94, 27-30.

Myrgorodska, I., Javelle, T., Meinert, C., Meierhenrich, U.J., (2017) Enantioresolution and quantification of monosaccharides by comprehensive two-dimensional gas chromatography. Journal of Chromatography A, 1487, 248-253.

Nedaei, S., Noori, A., Valipour, A., (2019) Effects of dietary galactooligosaccharide enriched commercial prebiotic on growth performance, innate immune response, stress resistance, intestinal microbiota and digestive enzyme activity in Narrow clawed crayfish (Astacus leptodactylus Eschscholtz, 1823). Aquaculture, 499, 80-89.

Nijman, R.M; Liu, Y.; Bunyatratchata, A.; Smilowitz, J.T.; Stahl, B.; D. Barile, (2018) Characterization and Quantification of Oligosaccharides in Human Milk and Infant Formula. Journal of Agricultural and Food Chemistry, 66, 6851−6859.

Ninonuevo, M.R., Perkins P.D., Francis, J., Lamotte, L.M., LoCascio, R.G., Freeman, S.L., Mills, D.A., German, J.B., Grimm, R., Lebrilla, C.B., (2008) Daily variations inoligosaccharides of human milk determined by microfluidic chips and massspectrometry, Journal of Agricultural and Food Chemistry, 56, 618–626.

Olano-Martín, E., Mountzouris, K. C., Gibson, G. R., Rastall, R. A., (2001) Continuous production of pectic oligosaccharides in an enzyme membrane reactor. Journal of Food Sciences, 66, 966-971.

Ostlund R, Sherman W (1996). Pinitol and derivatives thereof for the treatment of metabolic disorders. Patent reference WO/1996/029063.

Perguino, G., Trincone, A., Rossi, M., Moracci, M., (2004) Oligosaccharides Synthesis by glycosynthases. Trends in Biotechnology, 22, 31.

Petherick, A., (2010) Mother's milk: A rich opportunity. Nature, 468 (7327) S5-S7.

Rastall, R.A., (2010) Functional Oligosaccharides: Application and Manufacture. Annual Review of Food Science and Technology, 1, 305-339.

Ricardi, R.C., Menezes, E.W., Benvenutti, E.V., Schöffer, J.N., Hackenhaar, C.R., Hertz, P.F., Costa, T.M.H, (2017) Highly stable novel silica/chitosan support for β-galactosidase immobilization for application in dairy technology. Food Chemistry, 246, 343-350.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.J., Leotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., y Meheust, A., (2010) Prebiotic effects: metabolic and health benefits. British Journal of Nutrition. 104, S1-S63.

Robinson, R.C.; Colet, E.; Tian, T.; Poulsen N.A.; Barile, D., (2018) An improved method for the purification of milk oligosaccharides by graphitised carbon-solid phase extraction. International Dairy Journal, 80, 62-68.

Rodríguez-Sánchez, S., Hernández, O., Ruiz-Matute, A.I., Sanz. M.L., (2011) A derivatization procedure for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates by GC-MS in mulberry (Morus sp.) Food Chemistry 126, 353-359

Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M., (2009) Oligosaccharide analysis by graphitized carbon liquid chromatography-mass spectrometry. Analytical and Bioanalytical Chemistry, 394 (1), 163-174.

Ruiz-Aceituno, L., Rodriguez-Sanchez, S., Ruiz-Matute, A.I., Ramos, L., Soria, A.C. Sanz, M.L., (2013) Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts. Journal of the Science of Food and Agriculture 93 (11) 2797-2803.

Ruiz-Matute, A.I.; Ramos, L.; Martinez-Castro, I., Sanz, M.L., (2008) Fractionation of honey carbohydrates using pressurized liquid extraction with activated charcoal. Journal of Agricultural and Food Chemistry, 56 (18) 8309-8313.

Ruiz-Matute, A.I., Sanz, M.L., Corzo, N., Martin-Alvarez, P.J., Ibanez, E., Martinez-Castro, I., Olano, A., (2007) Purification of Lactulose from Mixtures with Lactose Using Pressurized Liquid Extraction with Ethanol-Water at Different Temperatures Journal of Agricultural and Food Chemistry, 55, 3346-3350.

Ruiz-Matute, A., Soria, A.C., Martinez-Castro, I., Sanz, M.L., (2007) A new methodology based on GC-MS to detect honey adulteration with commercial syrups. Journal of Agricultural and Food Chemistry, 55, 7264-7269.

Ruiz-Matute, A.I., Hernández-Hernández, O., Rodríguez-Sánchez, S., Sanz, M.L., Martínez-Castro, I., (2011) Derivatization of carbohydrates for GC and GC-MS analyses. Journal of Chromatography B, 879, 1226-1240.

Sabater, C., Prodanov, M., Olano, A., (2016) Quantification of prebiotics in commercial infant formulas. Food Chemistry, 194, 6-11.

Sako, T.; Matsumoto, K.; Tanaka, R., (1999) Recent progress on research and applications of non-digestible galacto-oligosaccharides Conferencia: 9th European Congress on Biotechnology (ECB9) Brussels, Belgium. International Dairy Journal,9 (1) 69-80.

Sanz, M.L., Martínez-Castro, I. (2007) Recent developments in sample preparation for chromatographic analysis of carbohydrates. Journal of Chromatography A, 1153 (1-2), 74-89.

Sanz, M.L., Ruiz-Matute, A., Corzo, N., y Martínez-Castro, I., (2009) Analysis of Prebiotic Oligosaccharides, en Prebiotics and Porbiotics Science and technology, Editores D. Charalampopoulos y R.A. Rastall, Springer: New York. 465-534.

Sanz, M.L., Sanz, J., and Martínez-Castro, I., (2002) Characterization of O-trimethylsilyl oximes of disaccharides by gas chromatography-mass spectrometry. Chromatographia, 56, 617.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., y Hutkins, R.W., (2006) Prebiotic Galactooligosaccharides Reduce Adherence of Enteropathogenic Escherichia coli to Tissue Culture Cells. Infection and Immunity. 74, 6920-6928.

Yasuno, S., Kokubo, K., Kamei, M., (1999) New Method for Determining the Sugar Composition of Glycoproteins, Glycolipids, and Oligosaccharides by High-performance Liquid Chromatography. Bioscience, Biotechnology, and Biochemistry. 63(8), 1353-1359 .

Splechtna B, Petzelbauer I, Baminger U, Haltrich D, Kulbe K, Nidetzky B (2001). Production of a lactose-free galacto-oligosaccharide mixture by using selective enzymatic oxidation of lactose into lactobionic acid. Enzyme and Microbial Technology 29(6–7): 434-440.

Tao, N., DePeters, E.J., Freeman, S., German, J.B., Grimm, R., (2008) Bovine milk glycome. Journal of Dairy Science, 91, 3768-3778.

Tao, N., Ochonicky, K.L., German, J.B., Donovan, S.M., Lebrilla, C.B., (2010) Structural determination and daily variations of porcine milk oligosaccharides, Journal of Agricultural and Food Chemistry, 58, 4653-4659.

Thomson, P., Medina, D.A., Garrido, D. (2008) Human milk oligosaccharides and infant gut bifidobacteria: Molecular strategies for their utilization. Food Microbiology, 75, 37-46.

Torres, P., Batista-Viera, F., (2012) Improved biocatalysts based on Bacillus circulans β-galactosidase immobilized onto epoxy-activated acrylic supports: applications in whey processing. Journal of Molecular Catalysis B: Enzymatic, 83, 57-64.

Tundis, R., Loizzo, M. R., Bonesi, M., (2018) Concentration of Bioactive Compounds from Elderberry (Sambucus nigra L.) Juice byNanofiltration Membranes. Plant Foods for Human Nutrition, 73 (4) 336-343.

Urakami, H., Saeki, M., Watanabe, Y., (2018) Isolation and assessment of acidic and neutral oligosaccharides from goat milk and bovine colostrum for use as ingredients of infant formulae. International Dairy Journal, 83, 1-9.

Venema, K., (2012) Intestinal fermentation of lactose and prebiotic lactose derivatives, including human milk oligosaccharides. International Dairy Journal, 22 (2) 123-140.

Verzele, M., Simoens, G., Van Damme, F., (1987) A critical review of some liquid chromatography systems for the separation of sugars. Chromatographia, 23, 292.

Wang, W. T., Norman C. L., Ackerman, B., Sweeley, C.C., (1984) Structural characterization of oligosaccharides by high-performance liquid chromatography, fast-atom bombardment-mass spectrometry, and exoglycosidase digestion. Analytical Biochemistry, 141, 366-381.

Wu, S., Grimm, R., German, J.B., Lebrilla, C.B., (2011) Annotation and Structural Analysis of Sialylated Human Milk Oligosaccharides. Journal of Proteome Research, 10, 856–868.

Wu, S., Grimm, R., German, J.B., Lebrilla, CB (2011) Annotation and Structural Analysis of Sialylated Human Milk Oligosaccharides. Journal of Proteome Research, 10 (2), 856-868.

Xue, Z., Han, Q., Gang, X. (2018) Altered brain metabolites in patients with diabetes mellitus and related complications - evidence from H-1 MRS study. Bioscience Reports, 38.

Yan, R.Y., Wang, H.Q., Liu, C., Kang, J., Chen, R.Y., (2013) α-Glucosidase-inhibitory iminosugars from the leaves of Suregada glomerulata. Bioorganic and Medicinal Chemistry, 21, 6796-6803.

Yoon, S. H., Mukerjea, R., Robyt, J. F., (2003) Specificity of yeast (Saccharomyces cerevisiae) in removing carbohydrates by fermentation. Carbohydrate Research, 338, 1127-1132.

Zhang, L., Su, Y., Zheng, Y.J., Shi, J.F., Zhu, Y.Y., Jiang, Y.J., (2010) Sandwich-structured enzyme membrane reactor for efficient conversion of maltose into isomaltooligosaccharides. Bioresource Techonology, 101 (23) 9144-9149.

Zotou, A., (2012) An overview of recent advances in HPLC instrumentation, Central Auropean Journal of Chemistry 10 (3) 554-569.

2. OBJETIVOS, METODOLOGÍA Y PLAN DE TRABAJO

No juzgues cada día por la cosecha que recoges, sino por las semillas que plantas.

Robert Louis Stevenson

2. OBJETIVOS, METODOLOGÍA Y PLAN DE TRABAJO

2.1. Objetivos

En la actualidad, existe un gran interés por el estudio de compuestos bioactivos que, al ser incorporados a alimentos, permitan que estos no solo satisfagan las necesidades nutricionales básicas, sino que proporcionen beneficios para la salud del consumidor o reduzcan el riesgo de enfermedad. Entre dichos compuestos se encuentran los carbohidratos bioactivos generalmente presentes como mezclas complejas con diferentes grados de polimerización (DP), unidades monoméricas y enlaces glicosídicos. Además, dichas mezclas, ya sea en matrices naturales o en mezclas de síntesis, normalmente están acompañadas de otros carbohidratos mayoritarios que pueden interferir en sus propiedades bioactivas o en la evaluación de las mismas, como puede ser el caso de la lactosa en la leche de cabra. Por tanto, resulta de especial interés el desarrollo de nuevas metodologías que permitan la identificación y cuantificación de carbohidratos bioactivos. Dichas metodologías deben ir orientadas: (i) al desarrollo de nuevos métodos de fraccionamiento que sean robustos, eficaces y fácilmente escalables, para la eliminación de carbohidratos mayoritarios interferentes, permitiendo el análisis de los compuestos minoritarios de interés; (ii) a la búsqueda de métodos analíticos que permitan una mayor resolución de los analitos que, dada su similitud estructural generalmente con los métodos existentes en la actualidad, es baja y (iii) a la identificación estructural mediante el acoplamiento a MS.

De acuerdo con estas consideraciones, el objetivo general de esta tesis, enmarcada dentro del proyecto AGR2011-7626 financiado por la Junta de Andalucía, se ha basado en **el desarrollo de nuevos métodos de análisis de carbohidratos bioactivos, en concreto, de oligosacáridos de leche de cabra y de mezclas comerciales de carbohidratos prebióticos**.

Para conseguirlo se plantearon los siguientes objetivos parciales:

- Evaluar el potencial de la combinación de β-galactosidasas y levaduras para la eliminación de lactosa de la leche de cabra, preservando los oligosacáridos propios de la leche y minimizando la formación de nuevos galactooligosacáridos.

- Desarrollar un método mediante GC-MS para el análisis de los trisacáridos de la leche de cabra.

- Desarrollar una nueva metodología mediante LC-MS que permita llevar a cabo el análisis cuali- y cuantitativo de oligosacáridos de la leche de cabra.

- Determinar la composición de oligosacáridos de calostro de cabra y su evolución durante el periodo de lactación.

- Establecer relaciones entre la estructura de los carbohidratos y los datos cromatográficos y espectrométricos. Aplicación al análisis de trisacáridos.

- Evaluar el potencial de la LC×LC para el análisis de carbohidratos bioactivos. Desarrollar un nuevo método que mejore la separación obtenida mediante LC monodimensional.

2.2. Metodología y plan de trabajo

Para alcanzar los objetivos parciales mencionados, se planteó el plan de trabajo que se muestra en la Figura 2.1., incluyendo dos grandes bloques: (i) el análisis de oligosacáridos de leche de cabra (**Sección 3**) y (ii) avances en la evaluación del comportamiento cromatográfico y espectrométrico en LC-MS para el análisis de carbohidratos prebióticos (**Sección 4**). A su vez, cada uno de estos bloques está constituido por distintas subsecciones, correspondientes a artículos ya publicados en revistas de alto índice de impacto o en proceso de publicación.

A continuación, se detalla la metodología seguida en cada una de estas secciones:

- **Sección 3: Análisis de oligosacáridos de leche de cabra**

- **Sección 3.1.** Desarrollo de un nuevo método de fraccionamiento basado en el empleo de enzimas y levaduras
  - Selección de β-galactosidasas para la eliminación de la lactosa
  - Optimización de condiciones experimentales del tratamiento con enzimas para calostro de cabra y lactosa patrón empleada como referencia
  - Evaluación del empleo de levaduras para la eliminación de glucosa galactosa
- **Sección 3.2.** Desarrollo de un método mediante GC-MS para el análisis de oligosacáridos de leche de cabra.
  - Selección del programa de temperaturas del GC
  - Evaluación y selección de los agentes derivatizantes y las condiciones de reacción óptimas
  - Validación del método
  - Aplicación del método al análisis de carbohidratos presentes en calostro de cabra
- **Sección 3.3.** Desarrollo y aplicación de una nueva metodología para el análisis cuali- y cuantitativo por LC-MS de los oligosacáridos de la leche de cabra:
  - Desarrollo de un método mediante HILIC-MS para el análisis cuantitativo de los oligosacáridos del calostro de cabra
  - Identificación exhaustiva de los oligosacáridos del calostro de cabra mediante nano-LC-chip-QToF MS
  - Aplicación a distintas muestras de calostro
  - Análisis cuali- y cuantitativo de los oligosacáridos de la leche de cabra durante el periodo de lactación

- **Sección 4: Avances en la evaluación del comportamiento cromatográfico y espectrométrico en LC-MS para el análisis de carbohidratos prebióticos**
    - **Sección 4.1.** Evaluación del comportamiento cromatográfico de di- y trisacáridos en LC-MS^n
        - Estudio de los parámetros cromatográficos (anchura, simetría, resolución, etc.) para carbohidratos con distinto composición monomérica y enlace glicosídico empleando distintos modos de operación
        - Selección de posibles iones marcadores de los espectros de MS^2 para dichos compuestos. Establecimiento de relaciones de los espectros con la estructura química de los compuestos
        - Aplicación al análisis de trisacáridos
    - **Sección 4.2.** Desarrollo de un método para separar mezclas de di- y trisacáridos mediante LC×LC y su aplicación a carbohidratos prebióticos
        - Derivatización de carbohidratos
        - Selección de fases estacionarias en LC monodimensional
        - Optimización de condiciones cromatográficas y modulación en LC×LC
        - Aplicación al análisis de mezclas complejas de oligosacáridos prebióticos

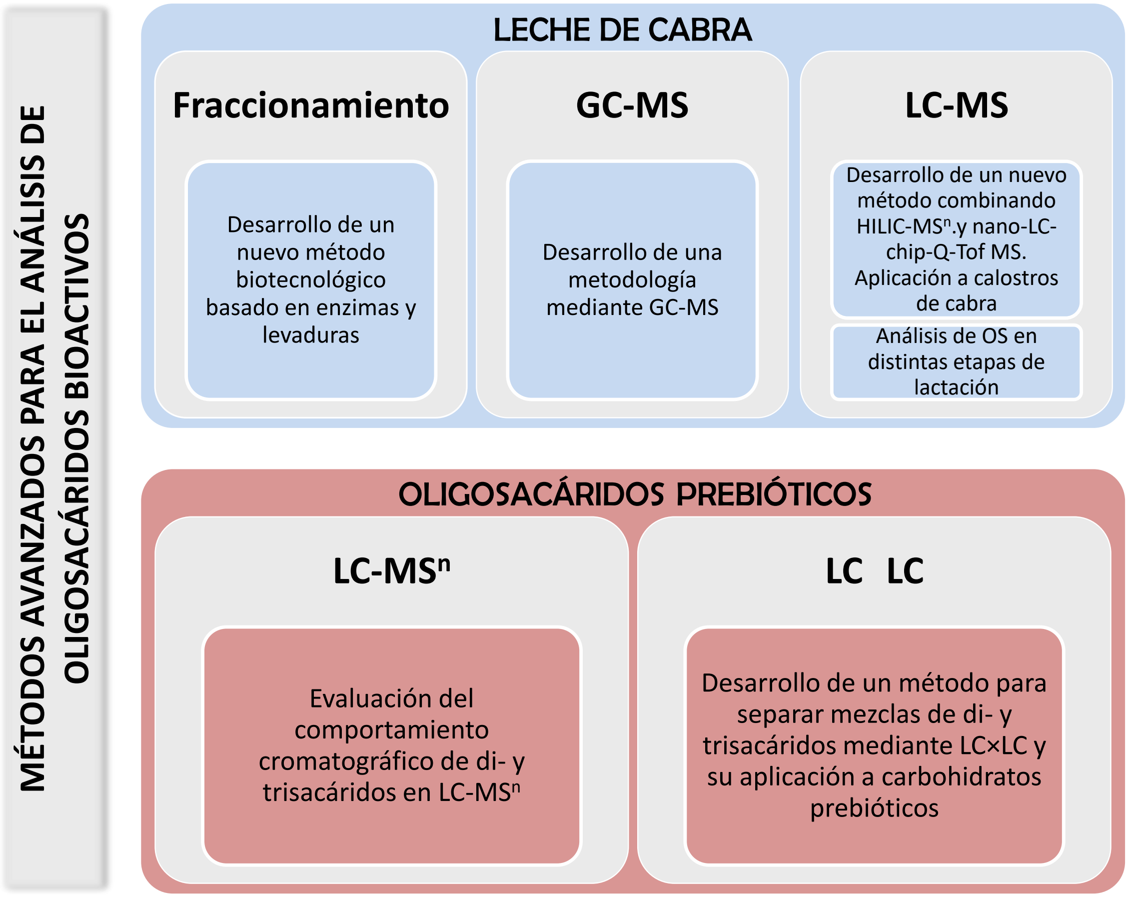

Figura 2.1. Plan de trabajo.

3. ANÁLISIS DE OLIGOSACÁRIDOS DE LECHE DE CABRA

La ignorancia afirma o niega rotundamente,
la ciencia duda.

Voltaire

3. PREFACIO

La leche de cabra es un alimento nutricionalmente completo y beneficioso para la salud, siendo menos alergénica y más digestible que las leches de otros mamíferos (Oliveira y col., 2015) y al igual que en estas leches, la lactosa es su carbohidrato más abundante (45-50 g L^{-1}) (Martínez-Ferez y col., 2006). Muchas de las propiedades beneficiosas para la salud de la leche (Figura 3.1) tradicionalmente se han relacionado con sus proteínas, péptidos y ácidos grasos aunque, más recientemente, también se han asociado a su contenido en OS.

Los **péptidos bioactivos** juegan un papel importante en el desarrollo y mantenimiento del metabolismo y de procesos inmunológicos y fisiológicos, lo que contribuye al desarrollo de productos funcionales en la industria láctea (Abeijón-Mukdsi y col., 2013; Assis y col., 2016).

Dentro de las **proteínas**, la αs1-caseína y la β-lactoglobulina son las principales responsables de las alergias a la leche, sin embargo en el caso de la leche de cabra tanto el tamaño de sus micelas como el contenido en αs1-caseína es menor que en la de vaca, lo que las hace responsables de una mejor digestibilidad y de disminuir la capacidad alergénica (Fiocchi y col., 2010; Koletzko y col., 2012).

La **fracción grasa** de la leche de cabra se presenta en forma de glóbulos de cadena más pequeña que en la de vaca, uno de los factores que incrementa su digestibilidad (Verruck y col., 2019). Está compuesta principalmente por triglicéridos (~98%) pero también existen otros compuestos en cantidades más pequeñas como fosfolípidos, colesterol, diacilgliceroles o ácidos grasos libres (Taylor y MacGibbon, 2011). Dentro de los ácidos grasos destaca el ácido linoleico, que juega un papel importante en el desarrollo y mantenimiento de procesos metabólicos e inmunológicos (Abeijón-Mukdsi y col., 2013; Assis y col., 2016). Los **conjugados del ácido linoleico (CLA)** se han descrito con actividad reconocida en la inhibición del cáncer, de la ateroesclerosis y de mejorar las funciones del sistema inmune (Elwood, y col., 2010).

Una ingesta completa y adecuada de **calcio** (Ca) es difícil de conseguir si no es a través del consumo de leche o derivados, en este sentido el consumo de leche se considera importante debido a su rico contenido en **minerales**. En 2009, Ceballos y col. demostraron que en la leche de cabra el contenido de Ca, P, Mg, Fe and Cu es más alto que en la de vaca, en concreto el contenido en Ca era de un 28,4% más que en la leche de vaca.

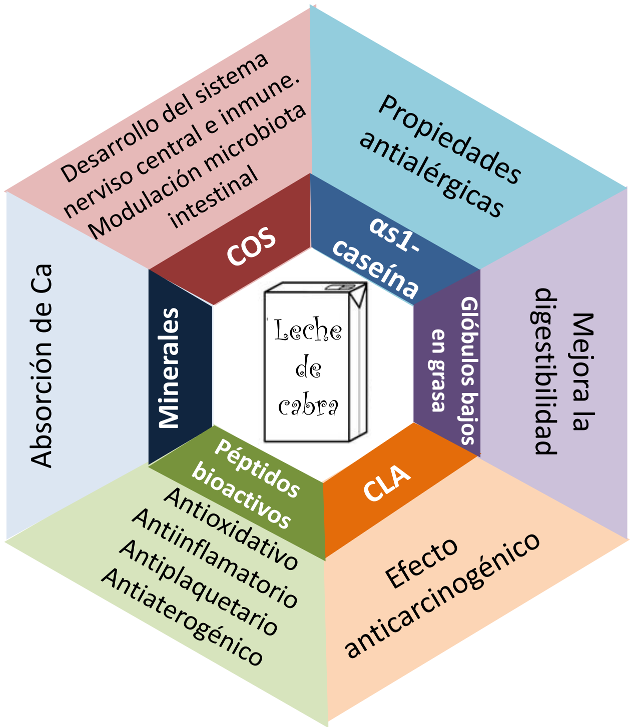

Figura 3.1: Resumen de las principales propiedades beneficiosas de la leche de cabra.

Los **oligosacáridos** de la leche de cabra (COS) presentan una gran complejidad y son, en comparación con los de leches de otros rumiantes, los OS más parecidos a los presentes en la leche humana, tanto desde el punto de vista estructural como en su concentración (Martinez-Ferez y col., 2006; Meyrand y col., 2013; Giorgio y col., 2018). Los COS se han descrito como responsables de efectos beneficiosos para la salud humana como el desarrollo del sistema nervioso central e inmune del neonato (Mehra y Kelly, 2006), actuando además sobre la biota intestinal, con un efecto prebiótico (Martínez-Ferez y col., 2006b; Oliveira y col., 2012), efecto antiadherente de patógenos causantes de enfermedades gastrointestinales, como *Campylobacter jejuni* y *Escherichia coli* (Morrow y col. 2005; Newburg y col. 2005) o disminuyendo los síntomas de la diarrea (Newburg y col. 2005; Bode 2009). También se ha descrito que tienen un efecto antiinflamatorio en el intestino (Daddaoua y col. 2006; Lara-Villoslada y col. 2006), y mejoran la función de barrera de células epiteliales (Barnett y col., 2016). Es por ello por lo que los COS se postulan como candidatos muy prometedores para ser suplementados en formulaciones infantiles y ser usados como una alternativa natural a la leche humana, siempre y cuando se desarrollen estrategias eficaces para su aislamiento o enriquecimiento (Kunz y Rudloff, 2006; Meyrand, 2013).

Los COS se componen de diversas estructuras con distintos DP (entre 3 y 7) que, generalmente, constan de un extremo reductor con una molécula de lactosa y una cadena constituida por distintas unidades monoméricas (tanto neutras como ácidas) unidas por diferentes enlaces glicosídicos. En el Anexo 2 de esta memoria se muestran las estructuras más características de COS.

Los **oligosacáridos neutros**, al igual que en la leche humana, se encuentran formados por distintas unidades de Glc, Gal, Fuc y GlcNAc unidas a la lactosa. Adicionalmente, los COS pueden contener también *N*-acetilgalactosamina (GalNAc) (Mehra y Kelly, 2006). Los oligosacáridos neutros más abundantes de la leche de cabra son la 3´-galactosil-lactosa y la *N*-acetilglucosaminil-lactosa (30-50 mg L^{-1} y 20-40 mg L^{-1}, respectivamente; Meyrand y col., 2013). Ambos oligosacáridos se encuentran en la leche de cabra en concentraciones muy superiores a las encontradas en leches de otros mamíferos domésticos como la vaca y oveja (Martinez-Ferez y col., 2006; Oliveira y col., 2015). Además, y a diferencia de la leche bovina y ovina, se ha descrito la presencia de oligosacáridos fucosilados en la leche de cabra, aunque los estudios existentes sobre dichos OS son escasos. Es, por tanto, necesario llevar a cabo nuevos estudios que profundicen en el conocimiento de la composición de los COS para poder establecer relaciones entre su estructura química y sus propiedades bioactivas y evaluar sus similitudes o diferencias con los OS presentes en la leche humana (Oliveira y col., 2015).

En relación a la **fracción ácida** de los COS, éstos presentan una o más unidades de ácido siálico (NeuAc) y, a diferencia de la leche humana, residuos de ácido *N*-glicolilneuramínico (NeuGc) (Meyrand y col., 2013). Los OS ácidos mayoritarios en la leche de cabra son la 3´-sialil-lactosa y la 6´-sialil-lactosa (Martínez-Férez y col., 2006) y, al igual que los OS neutros, diferentes factores como la raza, número de partos, dieta, periodo de lactación, presencia de genes específicos, etc. pueden influir en su composición (Mehra y Kelly, 2006; Meyrand y col., 2013; Claps y col., 2014; Foriga de Sousa y col., 2015). Los efectos biológicos de los OS ácidos se relacionan fundamentalmente con la presencia del ácido siálico y son de especial relevancia debido a su participación en el desarrollo cerebral y cognitivo del neonato (Wang, 2003) y en el desarrollo del sistema inmune postnatal (Kunz y Rudloff, 2006; Giorgio y col., 2018).

En la Tabla 3.1 se resumen los distintos OS identificados, hasta el momento, en la leche de cabra. A pesar de que se han identificado hasta 37 estructuras diferentes de COS (Meyrand y col., 2013) en cantidades comprendidas entre 250-300 mg L^{-1} (Martínez-Férez y col., 2006; Mehra y Kelly., 2006), dista mucho de la alta concentración (12-13 g L^{-1}) y de la gran diversidad de estructuras descritas para los HMOs (75 estructuras descritas por Meyrand y col., (2013) y 115 por Urashima y col., (2013)). Sin embargo, en comparación con leches de otros rumiantes, los COS superan unas 4 veces el contenido descrito en leche bovina y unas 10 veces el de la leche de oveja, presentando también una mayor variedad de estructuras (Urashima y col., 2013).

Los oligosacáridos de la leche son producidos únicamente en la glándula mamaria y se ha descrito que su composición varía significativamente, tanto cualitativa como cuantitativamente, durante el periodo de lactación (Martín-Sosa y col., 2003; Newburg y col., 2013; Balogh y col., 2015), lo que resulta relevante en la defensa y el desarrollo postnatal del individuo (Boehm y Moro, 2008). Aunque existen numerosos estudios relativos a los cambios producidos en los OS tanto de leche humana como bovina (Martín-Sosa y col., 2003; Fong y col., 2011; Newburg, 2013), la información sobre la evolución del perfil de oligosacáridos de la leche de cabra a lo largo del periodo de lactación es escasa y se limita al estudio de algunos COS, en concreto, ácidos como la 3’-sialil-lactosa, 6’-sialil-lactosa y disialil-lactosa (Claps y col., 2016), NeuAc y NeuGc (Formiga de Sousa y col., 2015). Recientemente, Marziali y col (2018) han estudiado la evolución de OS, tanto ácidos como neutros, tales como galactosil-lactosa, 3’-sialil-lactosa, 6’-sialil-lactosa, sialil-lactosamina y glicolil-neuraminil-lactosamina. Estos carbohidratos disminuyen drásticamente tras 48h de lactación, presentando una reducción de hasta un 70% a los 4 días de lactación.

Tabla 3.1: Lista de oligosacáridos de leche de cabra descrita por Meyrand y col., 2013. Código que indica el número de unidades de hexosa, fucosa, *N*-acetil hexosamina, ácido *N*-acetil neuramínico y ácido *N*-glicolil neuramínico de cada compuesto. [b] concentración no descrita en la bibliografía.

Código	¿OS neutro (N) o ácido (A)?	Masa (Da)	Conc. descrita (g L^{-1})	Nombre (abreviatura)
21000	N	488,174	n.d[b]	α-2'-Fucosil-lactosa (2'-FL)
30000	N	504,169	0,03-0,05	α-3'-Galactosil-lactosa (3-GL)
30000	N	504,169	n.d[b]	β-6'-Galactosil-lactosa (6-GL)
11100	N	529,201		Fucosil-lactosamina
20100	N	545,196	0,02-0,04	6'-*N*-Acetil-glucosaminil-lactosa (NAL)
20010	A	633,212	0,05-0,07	6'-Sialil-lactosa (6-SL)
20010	A	633,212	0,03-0,05	3'-Sialil-lactosa (3-SL)
20001	A	649,206	0,04-0,06	6'-Glicolil-neuraminil-lactosa (NGL)
40000	N	666,222		
10110	A	674,238	n.d[b]	6'-Sialil-lactosamina
10101	A	690,233		Glicolil-neuraminillactosamina
30100	N	707,248	Trazas	*N*-Acetil-glucosaminilhexosil-lactosa (NAHL)
20200	N	748,275	Trazas	Di-*N*-acetil-glucosaminil-lactosa (DNAL)
30010	A	795,264	Trazas	3'-Sialil-6'-galactosil-lactosa (3-SHL)
30010	A	795,264	Trazas	6'-Sialil-3'-galactosil-lactosa (6-SHL)
30001	A	811,259	Trazas	*N*-Glicolil-neuraminilhexosil-lactosa (SNGHL)
50000	N	828,275		
20110	A	836,291		Sialil-*N*-acetilglucosaminillactosa (SNAL)
31100	N	853,306	n.d[b]	Lacto-*N*-fuco-pentaosa III (LNFP III)
31100	N	853,306	n.d[b]	Lacto-*N*-fuco-pentaosa V (LNFP V)
40100	N	869,301	Trazas	*N*-Acetil-glucosaminildihexosil-lactosa (NADHL)
30200	N	910,328		
20020	A	924,307	0,001-0,005	Disialil-lactosa (DSL)
20011	A	940,302	Trazas	Sialil-*N*-glicolil-neuraminil-lactosa (SNGL)
20002	A	956,297	Trazas	Di-*N*-glicolil-neuraminil-lactosa (DNGL)
40010	A	957,317	Trazas	Sialil-di-hexosil-lactosa (SDHL)
60000	N	990,327		
40200	N	1072,381	0,001-0,005	Lacto-*N*-hexaosa (LNH)
30020	A	1086,360	Trazas	Disialil-hexosil-lactosa
30011	A	1102,355	Trazas	Sialil-*N*-glicolil-neuraminilhexosil-lactosa (SNGHL)
30300	N	1113,407		
30002	A	1118,350	Trazas	Di-*N*-glicolil-neuraminilhexosil-lactosa (DNGHL)
20400	N	1154,434		
41200	N	1218,438		
31300	N	1259,465		
40300	N	1275,460	Trazas	*N*-Acetil-glucosaminil-lacto-*N*-hexaosa (NALNH)
40210	A	1363,476	Trazas	Sialil-lacto-*N*-hexaosa (SNLH)

Como se ha comentado anteriormente, el perfil de OS parecido al de la leche humana hace de la leche de cabra un ingrediente nutricional muy prometedor. La multitud de funciones biológicas atribuidas a los OS de la leche parecen estar relacionadas con sus características estructurales únicas (Aquino y col., 2017). Sin embargo, debido a la gran complejidad estructural que presentan, con multitud de isómeros y posibilidades de unión de distintos monómeros a la lactosa y sus derivados, su caracterización no es tarea fácil y aún son muchos los COS que permanecen sin identificar.

La alta concentración de lactosa presente en la leche de cabra (45-50 g L^{-1}) en comparación con la de los COS (250-300 mg L^{-1}) presenta una dificultad en la caracterización de estos oligosacáridos (Mehra y Kelly., 2006), por lo que se hace necesaria una etapa previa de **fraccionamiento** o eliminación de este discárido.

Como se ha mencionado anteriormente en la introducción de esta memoria (sección 1.3.1), los métodos de fraccionamiento de carbohidratos más habituales se basan en el empleo de membranas, tratamientos con carbón activo, SEC y tratamientos microbiológicos o enzimáticos.

Las tecnologías de membrana como la filtración y diafiltración han sido bastante utilizadas para el fraccionamiento de carbohidratos de la leche de cabra gracias a los altos rendimientos de recuperación de COS que se obtienen. Sin embargo, muchas veces estos altos rendimientos se alcanzan a expensas de la pureza final de los COS recuperados (Aquino y col., 2017). A modo de ejemplo, la metodología desarrollada por Martínez-Ferez y col., (2006) empleando membranas cerámicas tubulares en la que se combinaba un proceso de ultrafiltración y de nanofiltración, permitió reducir en un 95% el contenido inicial de la lactosa original, sin embargo, se observó la degradación de algunos oligosacáridos durante el proceso. En cambio, en 2008, estos autores consiguieron recuperar el 99% de los oligosacáridos iniciales aumentando la presión de la membrana. Un enfoque similar fue utilizado por Oliveira y col. (2012) para la recuperación de los OS del suero de leche de cabra. El uso consecutivo de dos tipos de membranas de distinto tamaño de poro (25 kDa y 1 kDa) permitió la eliminación de las proteínas y glóbulos grasos pero dejando una elevada cantidad de lactosa remanente. El uso de una etapa posterior de fraccionamiento mediante SEC permitió la obtención de diferentes fracciones de COS libres de lactosa. Sin embargo, a pesar del éxito del proceso, resulta tedioso y costoso.

El uso de carbón activo para el fraccionamiento de los COS también ha sido descrito en la bibliografía. Urashima y col. (1991) utilizaron una columna de carbón activo junto con un proceso de diálisis, intercambio iónico, cromatografía en papel y SEC para aislar 4 trisacáridos neutros de calostro de cabra. Más recientemente se ha descrito el uso de cartuchos de carbón grafitizado para la obtención de una fracción enriquecida en COS previo a su análisis (Meyrand y col., 2013). Los cartuchos fueron en primer lugar activados con ACN y TFA y reequilibrados en agua. Se ha descrito que, en comparación con el método de lavado clásico con agua, el uso de estos disolventes disminuye la retención de la lactosa, mejorando su eliminación y aumentando la proporción de fase estacionaria disponible para la retención de los oligosacáridos (Robinson y col., 2018).

El empleo de enzimas con actividad β-galactosidasa ha sido comúnmente usado para la reducción del contenido en lactosa en distintos tipos de leches para su consumo por personas intolerantes o con malas digestiones (Zhu y col., 2018). Este tipo de fraccionamiento puede emplearse tanto a nivel analítico como preparativo, y presenta grandes ventajas en cuanto a la sencillez del proceso y escaso manejo de la muestra, así como a su posibilidad de escalado (empleo de reactores). Sin embargo, existen muy pocos estudios al respecto en leche de cabra. Recientemente, Aquino y col. (2017) han desarrollado una metodología para la obtención de fracciones enriquecidas en COS, basada en la hidrólisis enzimática de la lactosa mediante una lactasa de *Aspergillus oryzae* previa al proceso de concentración con membranas. Mediante este proceso, los monosacáridos generados son fácilmente eliminados obteniendo un 75% de recuperación de los COS presentes en el permeado de suero de cabra inicial. No obstante, en este trabajo no se han tenido en cuenta las posibles reacciones de transgalactosilación que pudieran tener lugar durante este proceso, dando lugar a la formación de nuevos oligosacáridos que no estaban presentes en la leche original (Ruiz-Matute y col., 2012; Zhu y col., 2018). Por tanto, resultaría de gran interés desarrollar procesos enzimáticos para la eliminación de la lactosa, en los que se llegase a un compromiso entre la formación de GOS y la conservación de los oligosacáridos bioactivos presentes en la leche de cabra para su potencial uso como ingredientes alimentarios.

El **análisis** de los COS requiere del uso de metodologías analíticas avanzadas que permitan tanto su identificación como su cuantificación. La alta capacidad de separación de las técnicas cromatográficas y la información estructural adicional proporcionada por su acoplamiento a la espectrometría de masas (MS) son de especial utilidad a la hora de caracterizar mezclas complejas como es el caso de la fracción de los OS de la leche de cabra.

La GC es una técnica muy utilizada para el análisis de carbohidratos de bajo peso molecular debido a su alto poder de resolución, sensibilidad y selectividad. Sin embargo, y como se ha comentado en la introducción (sección 1.3.2.1), para poder analizar los carbohidratos, estos deben ser derivatizados previamente. Mientras que la derivatización en el caso de OS neutros resulta trivial, no ocurre lo mismo en el caso de carbohidratos ácidos o básicos. En el caso de los oligosacáridos de la leche de cabra, los estudios realizados mediante esta técnica son muy escasos y la mayoría se limita a la determinación de la composición monomérica tras una etapa previa de hidrólisis o metanolisis de los OS (Viverge y col., 1997; Meyrand y col., 2013). En estos casos, la GC aporta una información complementaria a otras técnicas pudiéndose estimar la concentración total de OS en base a sus monómeros, sin embargo, se pierde información sobre la estructura y composición individual de cada OS.

En cuanto a la LC, esta es la técnica más establecida para el análisis de oligosacáridos de leche de cabra debido a su alta versatilidad y múltiples modos de operación, y al no requerir, en principio, una etapa de derivatización previa.

La HPAEC-PAD ha sido muy utilizada para la caracterización de los OS de la leche de cabra (Martínez-Férez y col., 2008; Martínez-Férez y col., 2009; Oliveira y col., 2012). Sin embargo, debido a la complejidad de los perfiles obtenidos para los COS, la falta de patrones y la utilización de condiciones incompatibles con MS, dificulta mucho la identificación de compuestos desconocidos mediante esta técnica. El acoplamiento de LC a MS proporciona una caracterización mucho más completa, tanto desde el punto de vista cualitativo como cuantitativo de los oligosacáridos de la leche de cabra. El uso de MS^n resulta muy interesante para la caracterización de OS, gracias a la información estructural adicional que proporciona. Dong y col. (2016) realizaron un estudio de los OS libres y los *N*-glicanos liberados a partir de proteínas de suero de leche humana, leche bovina y leche de cabra mediante LC acoplada a un sistema híbrido de espectrometría de masas de trampa iónica-Orbitrap usando una columna de C_{18} y una de PGC a altas temperaturas. Los OS fueron previamente permetilados y los espectros de MS^2 se utilizaron para la elucidación detallada de la estructura de los distintos isómeros. Se pudieron observar diferencias entre los *N*-glicanos permetilados derivados de proteínas de suero extraídas de las diferentes fuentes de leche, presentando un perfil más complejo que el de los oligosacáridos libres. Además, los *N*-glicanos en la leche bovina estaban más sialilados que fucosilados, mientras que en leche de cabra y leche materna estaban altamente fucosilados. La MS en tándem de azúcares permetilados mostró una ventaja al distinguir los isómeros de enlace y ramificación. Por otra parte, Meyrand y col. (2013) utilizaron también una columna de PGC para la caracterización de OS de distintas leches de cabra empleando nano-LC-Chip-Q-TOF MS. Gracias al empleo de la espectrometría de masas en tándem se confirmaron las estructuras de unos 30 COS, algunos de ellos desconocidos hasta el momento. Sin embargo, son muchas las estructuras que aún quedan sin identificar y los estudios sobre la leche de cabra muy limitados, por lo que la búsqueda de fases estacionarias con selectividad alternativa y que permitan una mejor separación de los distintos isómeros es de gran interés.

Uno de los modos de operación más usados para el análisis de mezclas complejas de OS es la HILIC, ya que proporciona resoluciones apropiadas entre los diferentes isómeros y permite su acoplamiento a MS (Cheng y col., 2016; Yan y col., 2018; Juvonen y col., 2019). Sin embargo, hasta el momento no se ha aplicado al análisis y caracterización de los oligosacáridos de leche de cabra lo que podría ser de gran interés para intentar avanzar en el conocimiento y caracterización de estos compuestos.

Considerando estos antecedentes, el objetivo general de esta sección de la tesis se centró en el desarrollo de métodos para el fraccionamiento y análisis de oligosacáridos de leche de cabra. En concreto, la **Sección 3.1** está basada en el artículo titulado "Selective biotechnological fractionation of goat milk oligosaccharides" de Martín-Ortiz y col. aceptado en el International Dairy Journal, 94 (2019) 38-45. En dicho trabajo se optimiza un método basado en el empleo de enzimas β-galactosidasas y levaduras para la obtención de una fracción de COS con bajo contenido de lactosa y de otros GOS.

La **Sección 3.2** recoge el artículo titulado "Development of a gas chromatographic-mass spectrometric method for the analysis of goat milk oligosaccharides" de Martín-Ortiz y col. en redacción para su publicación a una revista de Química Analítica, en el que se optimiza un proceso de derivatización para el análisis de oligosacáridos de leche cabra mediante GC-MS. Por último, en la **Sección 3.3** se pone a punto, por primera, vez un método mediante HILIC-MS en combinación con nano-LC-chip-Q-TOF MS para el análisis de OS de calostro de cabra. Estos resultados se han publicado en el artículo titulado "Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry" de Martín-Ortiz y col. en la revista J. Chromatography A 1428 (2016) 143-153. Este método ha resultado también de utilidad para el estudio de la evolución de los oligosacáridos de la leche de cabra durante el periodo de lactación. Dichos resultados constituyen la **Sección 3.4** de esta Memoria y han sido publicados en la revista J. Agric. Food Chem. 65 (2017) 3523−3531, con el título "Changes in caprine milk oligosaccharides at different lactation stages analyzed by high performance liquid chromatography coupled to mass spectrometry" de Martín-Ortiz y col.

Bibliografía

Abeijón Mukdsi, M. C., Haro, C., González, S. N., & Medina, R. B. (2013) Functional goat milk cheese with feruloyl esterase activity. Journal of Functional Foods, 5(2), 801–809.

Aquino, L., de Moura Bell, J., Cohen, J.L., Liu, Y., Lee, H., de Melo Silva, V. L., Domizio, P., Conte Junior, C. A., Barile, D. (2017) Purification of caprine oligosaccharides at pilot-scale, Journal of Food Enginering, 214, 226-235.

Assis, P.O.A. de, Guerra, G.C.B., Araújo, D.F.S., Araújo Júnior, R.F. de, Machado, T.A.D.G., Araújo, A.A. Queiroga, R.C.R.E. (2016) Intestinal anti-inflammatory activity of goat milk and goat yoghurt in the acetic acid model of rat colitis. International Dairy Journal, 56, 45–54.

Balogh, R., Jankovics, P., Béni, S. (2015), Qualitative and quantitative analysis of N-acetyllactosamine andlacto-N-biose, the two major building blocks of human milkoligosaccharides in human milk samples by high-performance liquidchromatography–tandem mass spectrometry using a porous graphiticcarbon column. Journal of Chromatography A, 1422, 140–146.

Barnett, A.M., Roy, N. C., McNabb, W. C., Cookson, A. L., (2016) Effects of a semi − purified oligosaccharides − enriched fraction from caprine milk on barrier integrity and mucin production of co-culture models of the small and large intestinal epithelium. Nutrients, 8, 1−17.

Bode, L., (2009) Human milk oligosaccharides: prebiotics and beyond. Nutrition Reviews, 67 (11), S183-S191.

Boehm, G., Moro, G., (2008) Structural and Functional Aspects of Prebiotics. Journal of Nutrition, 138 (9), 1818S-1828S.

Ceballos, L. S., Morales, E. R., Adarve, G. T., Castro, J. D., Martínez, L., Sampelayo, M. R. S. (2009). Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. Journal of Food Composition and Analysis, 22, 322–329.

Cheng, L., Xu, Q., Yang, K., He, J., Chen, D., Du, Y., Yin, H., (2016) Annotation of porcine milk oligosaccharides throughout lactation by hydrophilic interaction chromatography coupled with quadruple time of flight tandem mass spectrometry. Electrophoresis, 37 (11), 1525-1531.

Claps, S., Di Napoli, M.A., Sepe, L., Caputo, A.R., Rufrano, D., Di Trana, A., Annicchiarico, G., Fedele, V., (2014) Sialyloligosaccharides content in colostrum and milk of two goat breeds. Small Ruminant Research, 121, 116–119

Claps, S.; Antonietta Di Napoli, M.; Rocchina Caputo, A.; Rufrano, D.; Sepe, L.; Di Trana, A., (2016) Factor affecting the 3′ sialyllactose, 6′ sialyllactose and disialyllactosecontent in caprine colostrum and milk: Breed and parity. Small Ruminant Research, 134, 8−13.

Daddaoua, A., Puerta, V., Requena, P., Martinez-Ferez, A., Guadix, E., de Medina, F.S., Zarzuelo, A., Suarez, M.D., Boza, J.J., Martinez-Augustin, O., (2006) Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis, Journal of Nutrition, 136 (3), 672-676.

Dong, X., Zhou, S., Mechref, Y., (2016) LC-MS/MS analysis of permethylated free oligosaccharides and N-glycans derived from human, bovine, and goat milk samples, Electrophoresis 2016, 37, 1532–1548.

Elwood, P.C., Pickering, J.E., Givens, D., Gallacher, J.E., (2010) The consumption of milk and dairy foods and the incidence of vascular disease and diabetes: An overview of the evidence. Lipids, 45(10), 925–939.

Fiocchi, A., Brozek, J., Schünemann, H., Bahna, S. L., Berg, A. V., Beyer, K., ... Vieths, S. (2010). World allergy organization (WAO) diagnosis and rationale for action against cow's milk allergy (DRACMA) guidelines. Pediatric Allergy and Immunology, 21(21), 1–125.

Fong, B., Ma, K., McJarrow, P., (2011) Quantification of bovine milk OS using liquid chromatography-selected reaction monitoring-mass spectrometry. Journal of Agricultural and Food Chemistry, 59, 9788 – 9795.

Formiga de Sousa, Y. R.; da Silva Vasconcelos, M. A.; Costa, R. G.; Anderson de Azevedo Filho, C.; Prado de Paiva, E.; de Cássia Ramos do Egypto Queiroga, R., (2015) Sialic acid content of goat milk during lactation. Livestock Science, 177, 175−180.

Giorgio, D., Di Trana, A., Claps, S., (2018) Oligosaccharides, polyamines and sphingolipids in ruminant milk. Small Ruminant Research, 160, 23–30.

Juvonen, M., Kotiranta, M., Jokela, J., Tuomainen, P., Tenkanen, M., (2019) Identification and structural analysis of cereal arabinoxylan-derived oligosaccharides by negative ionization HILIC-MS/MS. Food Chemistry, 275, 176-185.

Koletzko, S., Niggemann, B., Arato, A., Dias, J. A., Heuschkel, R., Husby, S., Vandenplas, Y., (2012) Diagnostic approach and management of cow's-milk protein allergy in infants and children. Journal of Pediatric Gastroenterology and Nutrition, 55(2), 221–229.

Kunz, C., Rudloff, S. 2006, Health promoting aspects of milk oligosaccharides. International Dairy Journal, 16, (11), 1341-1346.

Lara-Villoslada, F., Debras, E., Nieto, A., Concha, A., Galvez, J., Lopez-Huertas, E., Boza, J., Obled, C., Xuas, J. (2006) Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. Clinical Nutrition, 25, 477−488.

Martinez-Ferez, A. Guadix, E.M. Guadix, S., (2006) Recovery of caprine milk oligosaccharides with ceramic membranes. Journal of Membrane Science, 276, 23-30.

Martínez-Ferez, A., Rudloff, S., Guadix, A., Henkelb, C.A., Pohlentzc, G., Bozaa, J.J., Guadix, M.E., Kunz, C., (2006) Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. International Dairy Journal, 16, 173–181.

Martinez-Ferez, A., Zapata, J.E., Guadix, A., Almecija, M.C., Gomez, M., Guadix, E.M., (2009) Obtention of goat milk permeates enriched in lactose-derived oligosaccharides. Desalination, 245, 730-736.

Martín-Sosa, S.; Martín, M. J.; García-Pardo, L. A.; Hueso, P., (2003) Sialyloligosaccharides in human and bovine milk and in infant formulas: Variations with the progression of lactation. Journal of Dairy Science, 86, 52−59.

Marziali, S; Guerra, E; Cerdan-Garcia, C; Segura-Carretero, A; Caboni, MF; Verardo, V., (2018) Effect of early lactation stage on goat colostrum: Assessment of lipid and oligosaccharide compounds. International Dairy Journal, 77, 65-72.

Mehra, R., Kelly, P. (2006) Milk oligosaccharides: structural and technological aspects, International Dairy Journal, 16, 1334–1340.

Meyrand, M., Dallas, D.C., Caillat, H., Bouvier, F., Martin, P., Barile, D., (2013) Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize alpha(s1)-casein. Small ruminant research, 113, 411-420.

Morrow, AL; Ruiz-Palacios, GM; Jiang, X; Newburg, DS., (2005) Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. Journal of Nutrition, 135 (5), 1304-1307.

Newburg, D. S. Glycobiology of human milk. Biochemistry 2013, 78, 771−785.

Newburg, DS; Ruiz-Palacios, GM; Morrow, AL., (2005), Human milk glycans protect infants against enteric pathogens. Annual Review of Nutrition, 25, 37-58.

Oliveira, D.L., Wilbey, R.A., Grandison, A.S., Duarte, L.C., Roseiro, L.B., (2012) Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. International Dairy Journal, 24, 102−106.

Oliveira, DL; Wilbey, RA; Grandison, AS; Roseiro, LB. (2015), Milk oligosaccharides: A review. International Journal of Dairy Technology, 68 (3), 305-321.

Robinson, R., Colet, E., Tian, T., Poulsen, N. A., Barile, D., (2018) An improved method for the purification of milk oligosaccharides by graphitised carbon-solid phase. International Dairy Journal, 80, 62-68.

Ruiz-Matute, A.I., Corzo-Martinez, M., Montilla, A., Olano, A., Copovi, P., Corzo, N., (2012) Presence of mono-, di- and galactooligosaccharides in commercial lactose-free UHT dairy products. Journal of Food Composition and Analysis, 28 (2), 164-169.

Taylor, M.W., MacGibbon, A.K.H. (2011) Milk lipids: General characteristics. In J. W. Fuquay, P. F. Fox, & P. L. H. Mcsweeney (Eds.). Encyclopedia of dairy sciences (3:649–3:654). (2nd ed.). London, UK: Elsevier Ltd.

Urashima, T., Saito, T., Kimura, T., (1991) Chemical Structures of Three Neutral Oligosaccharides obtained from Horse (Thoroughbred) Colostrum. Biochemistry and Physiology, 100B (1) 177-183.

Urashima, T., Taufik, E., Fukuda, K., Asakuma, S., (2013) Recent Advances in Studies on Milk Oligosaccharides of Cows and Other Domestic Farm Animals. Bioscience Biotechnology and Biochemistry, 77 (3), 455-466.

Verruck, S., Dantas, A., Prudencioa, E.S., (2019) Functionality of the components from goat's milk, recent advances for functional dairy products development and its implications on human health. Journal of Functional Foods, 52, 243–257.

Viverge, D; Grimmonprez, L; Solere, M., (1997), Chemical characterization of sialyl oligosaccharides isolated from goat (Capra hircus) milk. Biochimica et Biophysica Acta-GEnetal Subjects, 1336 (2), 157-164.

Wang, B., Staples, A., Hunter, A., Yu, B., Brand Miller, J. (2003) Effect of experimental oligosaccharide on brain and body weight. Asia Pacific Journal of Clinical Nutrition, 12(Suppl), S60.

Yan, J., Ding, J., Jin, G., Yu, D., Yu, L., Lone, Z., Guo, Z., Chai, W., Liang, X., (2018) Profiling of Sialylated Oligosaccharides in Mammalian Milk Using Online Solid Phase Extraction-Hydrophilic Interaction Chromatography Coupled with Negative-Ion Electrospray Mass Spectrometry. Analytical Chemistry, 90 (5), 3174-3182.

Zhu, F; Prosser, C; Zhu, Y; Otter, D; Hemar, Y., (2018), Enzymatic formation of galactooligosaccharides in goat milk. Food Bioscience, 26, 38-41.

Sección 3.1: Fraccionamiento biotecnológico selectivo de oligosacáridos de leche de cabra

“Selective biotechnological fractionation of goat milk oligosaccharides “

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Selective biotechnological fractionation of goat milk carbohydrates

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ABSTRACT

Goat colostrum is a rich source of carbohydrates, mainly constituted by lactose, although several minor bioactive oligosaccharides are also present. Analysis of these caprine milk oligosaccharides (COS) is not straightforward, and usually requires a previous fractionation step to remove lactose. In this work, a biotechnological fractionation methodology based on the use of a β-galactosidase from *Kluyveromyces lactis* was optimised (pH, incubation time, goat milk:enzyme volume ratio) to hydrolyse lactose, preserving the COS profile. Best results were obtained after 15 min of enzymatic treatment using 0.68 U mL^{-1} of enzyme at 37 °C and pH 7. Efficient removal of resulting monosaccharides was finally carried out by the incubation of these samples with *Saccharomyces cerevisiae* (37 °C, 24 h). Fractionation of these carbohydrates could help to better determine COS structures and to expand the applications of the purified COS in the food and pharmaceutical industries.

1. Introduction

Currently, goat milk is the world's third most produced variety of milk (FAOstat, 2018). Its consumption is gaining great relevance considering the numerous beneficial properties associated to its constituents (Verruck, Dantas, & Prudencio, 2019), such as anti-inflammatory effects (Daddaoua et al., 2006), prebiotic activity (Oliveira, Wilbey, Grandison, Duarte, & Roseiro, 2012) or expression and development of brain and central nervous system functions (Mehra & Kelly, 2006), among others. Apart from lipids and proteins, goat milk is a rich source of carbohydrates, being lactose (galactosyl-β-D-(1 → 4)-glucose) the most abundant one (around 44 g L^{-1}) (Martínez-Ferez et al., 2006a). Moreover, several neutral and acidic minor oligosaccharides (OS), some of them similar to those found in human milk, are present (Meyrand et al., 2013).

As previously reported, caprine milk oligosaccharides (COS) have complex structures with different glycosidic linkages and degrees of polymerisation (between 2 and 7) (Martin-Ortiz et al., 2016; Meyrand et al., 2013). Monomeric units of glucose (Glc), galactose (Gal), *N*-acetylglucosamine or *N*-acetylgalactosamine (Hex*N*Ac) and fucose (Fuc) linked to a lactose core make up neutral COS, whereas these monomers plus *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) form acidic OS. These OS are found in goat milk at higher concentrations than in milks from other farm mammals (Martínez-Ferez et al., 2006a), especially in goat colostra, in which they are more abundant (Martin-Ortiz et al., 2017; Mehra & Kelly, 2006). Different bioactive properties have been recently attributed to COS, such as potential prebiotic activity (Oliveira et al., 2012), reduction of intestinal inflammation (Lara-Villoslada et al., 2006), and improvement of barrier function of epithelial cell co-cultures (Barnett, Roy, McNabb, & Cookson, 2016). Therefore, conveniently isolated COS could be a potentially promising functional food ingredient for improving intestinal health which could be used in different foodstuffs (e.g., infant milks).

Considering the high concentrations of lactose and the similar structure of the minor OS, the isolation of COS is challenging and not straightforward. Several methods for the selective fractionation of these carbohydrates have been proposed mainly based on the removal of monosaccharides by membrane-based techniques (Martínez-Ferez et al., 2009; Martínez-Ferez, Guadix, Zapata-Montoya, & Guadix, 2008) or on the use of size exclusion chromatography (SEC) to fractionate OS according to their degree of polymerisation (Martin-Ortiz et al., 2016, 2017). Among these techniques, SEC provides relatively good yields of OS (81–92%) with high degree of purity, although it has several disadvantages such as it is a time-consuming technique, the permeation gel used is

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expensive and the sample obtained is relatively diluted (Hernández, Ruiz-Matute, Olano, Moreno, & Sanz, 2009).

The use of microbiological treatments for carbohydrate fractionation has also been proposed. Among them, the use of *Saccharomyces cerevisiae* has been proposed as a clean and easily scalable biotechnological procedure to be applied for the removal of interfering low molecular weight carbohydrates (LMWC) from inositols-enriched legume extracts (Ruiz-Aceituno et al., 2013), honeys (Ruiz-Matute et al., 2007; Sanz, González, Lorenzo, Sanz & Martínez-Castro, 2005) and prebiotic oligosaccharide mixtures such as galactooligosaccharides (GOS) (Goulas, Tzortis & Gibson, 2007; Hernández-Hernández, Calvillo, Lebrón-Aguilar, Moreno, & Sanz, 2011) and fructooligosaccharides (Nobre et al., 2016). However, lactose is not metabolised by this particular yeast (Yoon, Mukerjea, & Robyt, 2003); therefore, it has not directly been applied to milk samples. On the other hand, *Kluyveromyces lactis* has been traditionally used as industrial yeast in dairy products, as it is generally recognised as a safe (GRAS) microorganism and has a good growth yield and a higher β-galactosidase activity as compared to other yeasts (Zolnere & Ciprovica, 2017).

The employment of enzymes as β-galactosidases (EC 3.2.1.23) is an attractive alternative to the carbohydrate fractionation and different applications of commercial β-galactosidases [e.g., Maxilact® (DSM, Netherlands), HA-lactase® (Christian-Hansen, Denmark)] have been efficient for the elaboration of some foods free of lactose such as ice-creams, milk drinks or dairy preparations designed for elderly people (Montilla, Megías-Pérez, Olano, & Villamiel, 2015; Ruiz-Matute et al., 2012). Remarkably, there have been efforts to transfer genes from *K. lactis* to *S. cerevisiae* to generate genetically modified yeast strain(s) with the ability to consume lactose (Rubio-Texeira, Arévalo-Rodríguez, Lequerica, & Polaina, 2000). Nevertheless, most of those strains exhibited unsuitable characteristics, such as genetic instability of the Lac phenotype or diauxic growth.

In this context, the approach based on the combination of a β-galactosidase enzyme exerting high hydrolytic activity with a yeast strain efficient in removing released monosaccharides could be a successful strategy for the fractionation of carbohydrate mixtures which are vastly dominated by lactose. de Moura Bell et al. (2016) have proposed the use of a β-galactosidase from *Aspergillus oryzae* to hydrolyse the lactose from bovine colostrum whey permeate and improve the selectivity and efficiency of membrane processes in the recovery of bioactive oligosaccharides.

However, to the best of our knowledge, applications of microbiological treatments to the removal of LMWC from goat milks are scarce. Some studies have evaluated the use of commercial β-galactosidases (from *K. lactis* and/or from *A. oryzae*) to promote the formation of new GOS (allolactose, 6'-galactobiose and 6'-galactosyl-lactose) in reconstituted goat milk; however, the content and preservation of COS was not evaluated and these studies were not carried out for fractionation purposes (Pruksasri & Supee, 2013; Zhu, Prosser, Zhu, Otter, & Hemar, 2018).

Only Aquino et al. (2017) have recently proposed a novel approach based on the use of a β-galactosidase from *A. oryzae*, followed by the treatment with *S. cerevisiae* and nanofiltration, for the purification at a large scale of oligosaccharides from goat milk. However, during these treatments special care should be taken with the degradation of the bioactive OS and the occurrence of transgalactosylation reactions, which can give to new OS of different composition. Aquino et al. (2017) stated that the β-galactosidase from *A. oryzae* did not degrade the 3'-sialyl-lactose, 6'-sialyl-lactose and 6'-sialyl-lactosamine, as previously assayed (de Moura Bell et al., 2016); however, the evolution of other OS and the potential production of new OS was not evaluated. As far as we know, the use of these treatments for the removal of lactose from goat colostrum, has not yet been carried out.

Therefore, in this work, a biotechnological procedure using a β-galactosidase from *K. lactis* (Lactozym® pure 6500 L) has been optimised to hydrolyse lactose from a pooled goat colostrum sample, keeping COS and minimising the formation of other OS by transgalactosylation reactions. The approach was completed using *S. cerevisiae* to remove the released galactose and glucose.

2. Materials and methods

2.1. Goat colostrum samples

For this study, colostrum from twelve individual Murciano-Granadina goats reared at Hermanos Archiduque farm (Granada, Spain) were kindly provided by Dr. A. Clemente (EEZ, CSIC; Spain). Samples were collected, pooled, aliquoted and immediately frozen at −80 °C until further analysis. Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line of corresponding European Directive (2010/63/EU). An experimental protocol was approved by the Ethics Committee for Animal Research from the Animal Nutrition Unit.

2.2. Enzyme and yeast

A commercial preparation β-galactosidase from *K. lactis* (Lactozym® Pure 6500 L HP G) was kindly supplied by Novozymes (Bagsvaerd, Denmark). The β-galactosidase activity as determined by the manufacturer was 6500 LAU g^{-1}. *S. cerevisiae* type II from Sigma Chemical Co. (St. Louis, MO, US) was activated with 1.5 mL of Milli-Q water at the selected incubation temperature (37 °C) for 30 min under stirring (160 rpm).

2.3. Carbohydrate standards

Glucose, lactose and 4′-galactosyl-lactose were acquired from Sigma-Aldrich. Galactooligosaccharides (GOS) synthesised according to Martínez-Villaluenga, Cardelle-Cobas, Corzo, Olano, and Villamiel (2008), using Lactozym 3000 L (Novozymes), were used as reference.

2.4. Fat and protein removal

Fat and proteins were removed from the samples following the methodology described by Martínez-Ferez, Guadix, and Guadix, (2006b) with small modifications. Briefly, samples were defatted by centrifugation at 6500 ×*g* for 15 min at 5 °C, then kept in an ice bath for 30 min and filtrated through Whatman Nº 1 filter paper to remove the supernatant lipid layer, which was discarded.

The total protein fraction was precipitated by adding two volumes of cold ethanol to the skimmed colostrum samples and shaking for 2 h in an ice bath. The solution was then centrifuged at 6500 ×*g* for 30 min at 5 °C and supernatant was carefully collected. Ethanol was evaporated from the sample in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37 °C and the remaining aqueous solution containing the carbohydrate fraction was frozen and lyophilised.

2.5. Enzymatic treatment

2.5.1. Effect of pH

To select the optimal conditions for lactose hydrolysis, 350 mg of lactose were dissolved in 5 mL of different buffers: (i) 0.1 M sodium acetate at pH 4.5, (ii) 0.1 M sodium phosphate at pH 7 and (iii) 0.1 M tris-(hydroxymethyl)-aminomethane-HCl (Tris-HCl) at pH 8. These solutions (995 μL) were mixed with 5 μL, corresponding to 0.34 U mL^{-1}, of β-galactosidase and incubated at 37 °C for 1 h under constant stirring using a Thermomixer at 600 rpm. Samples were taken at 0 and 1 h. After these times, the reaction was stopped at 100 °C for 1 min and samples were immediately cooled in ice.

2.5.2. Effect of incubation time and enzyme concentration

Colostrum carbohydrate fraction (375 mg) and lactose (350 mg) were independently dissolved in 5 mL of phosphate buffer at pH 7. These amounts were chosen according to previous assays that indicated that this lactose concentration was high enough to increase the cost-efficiency of the hydrolysis process without saturating the enzyme β-galactosidase (Diez-Municio, Montilla, Moreno, & Herrero, 2014). Of these solutions 985–997 μL were mixed with enzyme (3, 5, 10 and 15 μL, corresponding to 0.20, 0.34, 0.68 and 1.02 U mL^{-1}, respectively) to get a final volume of 1 mL. Incubations were carried out at 37 °C and under constant stirring. Aliquots of 100 μL were taken at 0, 1, 3, 5 and 8 h and the reaction was stopped as indicated before. Evolution of OS during enzymatic treatment was also studied at shorter incubation times using 5, 10 and 15 μL of enzyme (0.34, 0.68 and 1.02 U mL^{-1}), taking aliquots at 0, 15, 30 and 45 min. All samples were kept at −18 °C for further analyses. All assays were carried out in triplicate.

2.6. Yeast treatment

Yeast treatment was carried out as described by Ruiz-Aceituno et al. (2013). Goat colostrum and lactose control samples, obtained after incubation with β-galactosidase (0.68 U mL^{-1}) for 15 min, were treated with 1% (w/v) *S. cerevisiae* (Sigma-Aldrich) at 37 °C under stirring for 30 h. All assays were done in triplicate. Aliquots were taken just before yeast addition (time 0) and at 10 min, 2, 4, 6, 24 and 30 h of treatment. Then, they were centrifuged at 4400 ×*g* at 10 °C for 10 min and filtered through Whatman No. 4 filters to remove yeast, and kept at −20 °C until analysis.

2.7. Analysis by high performance anion exchange chromatography with pulsed amperometric detection

Prior to high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) lactose determination, samples were diluted 1/20 (v:v) using Milli-Q water. For OS analyses, samples were analysed without dilution. A ICS-2500 Dionex system (Dionex Corp., Sunnyvale, CA, USA) consisting of a GP50 gradient pump, an automatic injector and an ED50 electrochemical detector with a gold working electrode and a Ag/AgCl reference electrode was used. Separation of carbohydrates was carried out on a CarboPac PA 1 guard column (50 × 4 mm) and a CarboPac PA-1 anion-exchange column (250 × 4 mm) (Dionex Corp.). Different linear gradients using 150 mM sodium hydroxide (VWR, Barcelona, Spain) as solvent A and 50 mM sodium acetate (Merck, Madrid, Spain) in 150 mM sodium hydroxide as solvent B were tested. Optimal conditions for lactose analysis (ramp 1) were as follows: Eluent B was kept at 5% for 1 min, changed to 10% in 1 min, kept at these conditions for 3 min, changed to 40% in 1 min and kept constant for 5 min. After each run, the column was washed with 100% of eluent C (150 mM sodium hydroxide and 1 M sodium acetate) and re-equilibrated for 10 min at initial conditions; 20 μL of testing samples were injected. Optimal chromatographic conditions for the analysis of OS (ramp 2) were: Eluent B was kept at 5% for 1 min, changed to 10% in 1 min, kept at these conditions for 18 min, changed to 40% in 1 min and kept constant for 5 min. After each run, the column was washed with 100% of eluent C (150 mM sodium hydroxide and 1 M sodium acetate) and then, re-equilibrated for 10 min at initial conditions; several injection volumes (between 50 and 100 μL) of testing samples were assayed.

All eluents were degassed by flushing helium. In both cases, the flow rate was 1.0 mL min^{-1} and the column temperature 25 °C. Carbohydrates were detected using triple pulsed amperometry with the following potentials and durations: E1 = +0.15 V (t1 = 400 ms), E2 = +0.75 V (t2 = 200 ms), E3 = 0.6 V and E4 = −0.8 V (t3 = 200 ms). Acquisition and processing of data were carried out using the Chromeleon software version 6.7 (Dionex Corp., Sunnyvale, CA).

Quantitative analysis of monosaccharides (glucose + galactose), disaccharides (lactose, allolactose and trehalose) and oligosaccharides (GOS and COS) in colostrum samples was performed in triplicate using external standard calibration curves of glucose, lactose and 4′-galactosyl-lactose, respectively, within the range 0.001–2.0 mg mL^{-1}. Calibration curves were: glucose, $y = 638.43x + 2.9128$ ($R^2 = 0.9932$); lactose, $y = 348.05x + 10.78$ ($R^2 = 0.9874$); 4′-galactosyl-lactose, $y = 232.86x - 0.1037$ ($R^2 = 0.9970$). Results were expressed in mg g^{-1} of defatted and deproteinised colostrum sample.

2.8. Statistical analysis

For statistical analysis the Univariate analysis of variance (ANOVA) and Tukey test (SPSS software) were used. Differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1. Analysis of goat colostrum

Lactose and oligosaccharides from pooled goat colostrum could not be analysed in a single run, considering the differences in their respective concentrations. Therefore, two chromatographic methods (different dilutions of the samples, injection volumes and chromatographic conditions) were used as indicated in the Materials and methods section.

Fig. 1A shows the HPAEC-PAD profile of the carbohydrate fraction of pooled goat colostrum eluted using ramp 2 and 75 μL of injection volume. Under these chromatographic conditions, glucose + galactose (peak 1) and lactose (peak 2) were separated from COS (peaks 3 and 4), allowing the quantitation of these minor carbohydrates. All detected peaks were satisfactorily resolved with the exception of a coelution between lactose and a peak eluting at 3.8 min (labelled as 3a). By comparing this chromatographic profile with that of the reference GOS (obtained under conditions previously reported by Martínez-Villaluenga et al., 2008) and with those thoroughly described in the literature (Rodriguez-Colinas et al., 2011; Yin, Bultema, Dijkhuizen, & van Leeuwen, 2017), this peak was tentatively assigned as 6'-galactosyl-lactose (β-D-galactosyl-β-D-(1 → 6)-lactose). Unfortunately, this carbohydrate could not be quantified without interferences at goat colostrum. The presence of 6'-galactosyl-lactose had been previously detected in goat milk at low concentrations (Meyrand et al., 2013). Other peaks eluting between 5 and 7.5 min (peaks 3) were identified as trisaccharides, constituted by galactose and glucose units by comparison with the reference GOS sample and according to those reported in the literature (Cardelle-Cobas et al., 2009; Zolnere & Ciprovica, 2017). Considering that 3'- galactosyl-lactose is the most abundant trisaccharide previously detected in goat milks (Mehra & Kelly, 2006; Meyrand et al., 2013), peak 3b was tentatively assigned to this trisaccharide. These carbohydrates (peaks 3) were identified in this work as COSg. Peaks eluting between 8.8 and 11 min and between 16 and 21 min, only present in pooled goat colostrum, were

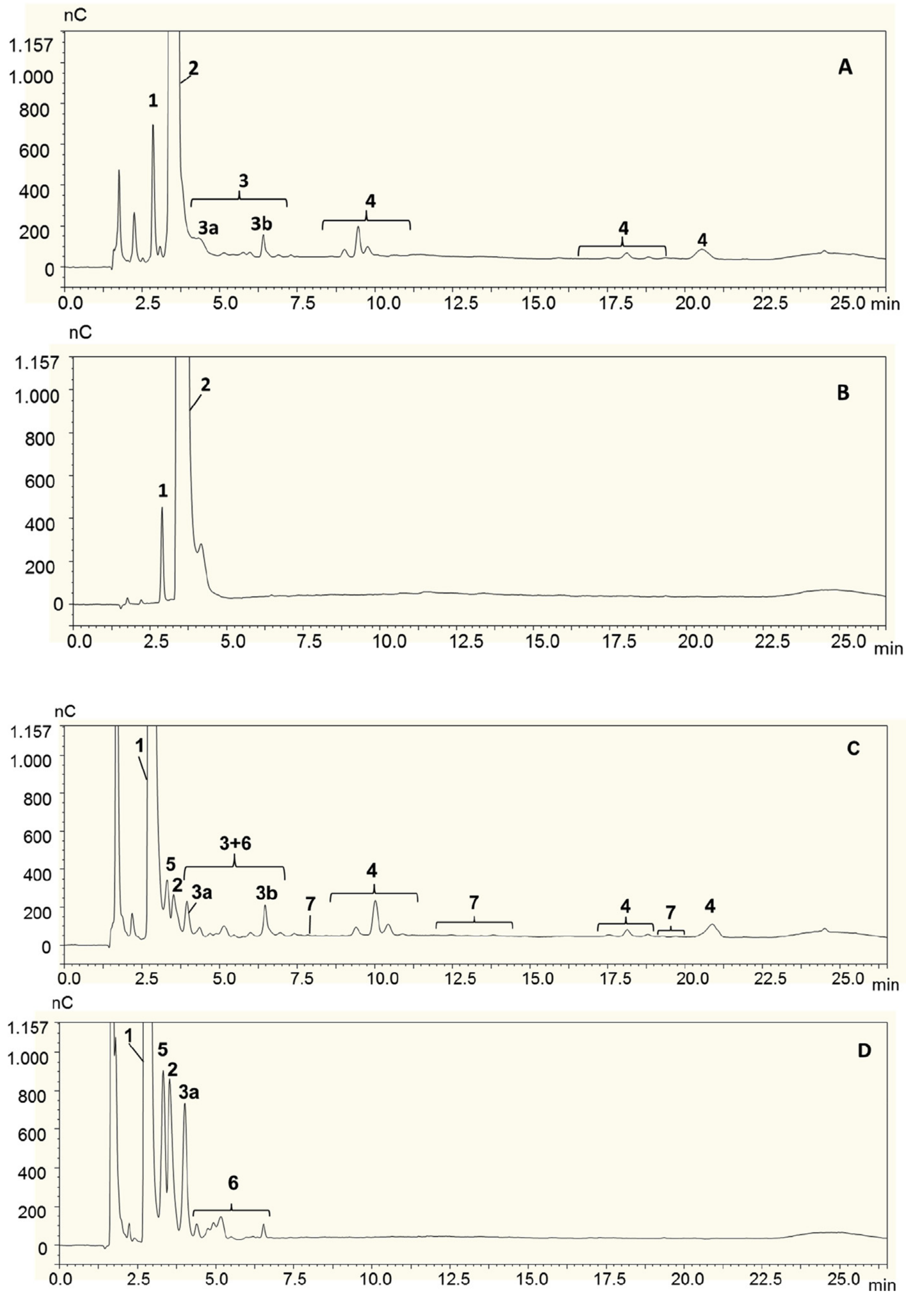

Fig. 1. HPAEC-PAD profile of carbohydrates present in samples before enzymatic treatment [(A) pooled goat colostrum and (B) lactose standard] and after 45 min of β-galactosidase incubation (0.68 U mL^{-1}) [(C) pooled goat colostrum and (D) lactose standard]. Peaks are: 1, glucose + galactose; 2, lactose; 3, COSg; 3a, 6'-galactosyl-lactose; 3b, 3'-galactosyl-lactose; 4, COSc; 5, allolactose; 6, GOS trisaccharides; 7, COSh.

assigned as COSc (peaks 4). A lactose standard solution (control) was also analysed under these conditions for comparative purposes (Fig. 1B).

3.2. Effect of pH conditions on lactose hydrolysis

Table 1 shows the percentages of resulting carbohydrates from the hydrolysis of lactose standard (75 mg mL^{-1}) by the action of β-galactosidase from *K. lactis* (0.34 U mL^{-1}) for 1 h at 37 °C using different buffer solutions (pH 4.5, 7 and 8). While lactose could hardly be hydrolysed at pH 4.5 and pH 8 (98.2% and 94.1% of lactose remaining), this reaction was successful at pH 7 (1.6% of lactose remaining). Minor amounts of different GOS were produced under these conditions. Therefore, 0.1 M sodium phosphate at pH 7 was selected for further studies. Martínez-Villaluenga et al. (2008) found that inactivation of β-galactosidase from *K. lactis* was at pH 5.5 and the optimal pH conditions for the hydrolysis of lactose were

Table 1
Percentage of carbohydrates obtained after incubation of lactose with β-galactosidase (*K. lactis*) for 1 h at 37 °C at different pH values.[a]

Carbohydrate	Percentage obtained		
	pH 4.5	pH 7	pH 8
Glucose + galactose	1.8 (0.1)	98.0 (0.1)	5.51 (0.05)
Lactose	98.2 (0.1)	1.60 (0.05)	94.1 (0.4)
Oligosaccharides	0.0	0.40 (0.03)	0.3 (0.2)

[a] Values are given with standard deviation in parentheses.

between 6.5 and 7.5. However, these conditions were also optimal for the production of GOS. Then, as one of the aims of this work was to minimise GOS production, evaluation of incubation time and enzyme concentration was further carried out.

3.3. Effect of incubation time and enzyme concentration on lactose hydrolysis

Once the pH conditions were chosen, the effect of incubation time and enzyme concentration was evaluated to select the most appropriate conditions to maximise lactose hydrolysis. Fig. 1C and D show the HPAEC-PAD profiles of the goat colostrum and the lactose standard (control), respectively, after incubation with 0.68 U mL^{-1} of β-galactosidase (*K. lactis*) for 45 min. As it can be observed, and comparing with Fig. 1A and B, lactose (peak 2) experimented a markedly decrease in these samples, while glucose + galactose (peak 1) noticeably increased.

Fig. 2 shows the changes of lactose content (%) experimented in both goat colostrum (Fig. 2A) and lactose control sample (Fig. 2B) during enzymatic treatment using different β-galactosidase concentrations. While a concentration of 0.20 U mL^{-1} of enzyme was not enough to hydrolyse the lactose present in the control and in the goat colostrum, even after 8 h of treatment (45 and 20% lactose remaining, respectively), better results were obtained using 0.34, 0.68 and 1.02 U mL^{-1} of enzyme, respectively. Under these conditions, concentrations of lactose lower than 5% after 1 h of β-galactosidase treatment were detected.

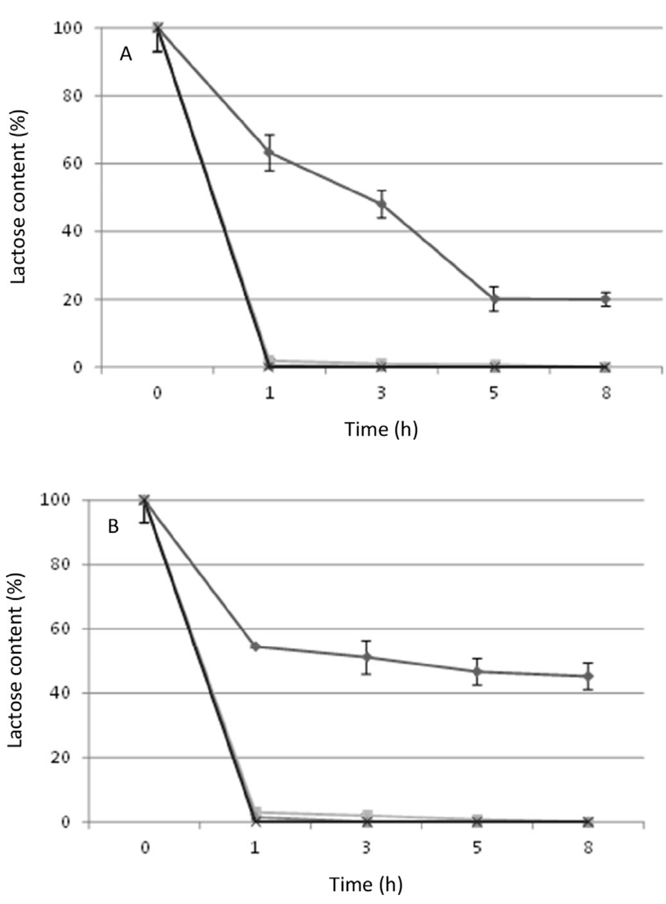

Fig. 2. Evolution of lactose content (%) during β-galactosidase treatment at long incubation times at pH 7 and using different concentrations of enzyme (◆, 0.20 U mL^{-1}; ■, 0.34 U mL^{-1}; ▲, 0.68 U mL^{-1}; ×, 1.02 U mL^{-1}) in pooled goat colostrum (A) and in lactose standard solution (B).

The efficiency of the lactose hydrolysis was then evaluated in both goat colostrum and lactose control samples at shorter reaction times (15, 30 and 45 min) using 0.34, 0.68 and 1.02 U mL^{-1} of enzyme, respectively (Fig. 3). While only 15 min of incubation were enough to remove more than 95% of lactose using 0.68 and 1.02 U mL^{-1} of enzyme, 60 min of treatment were required to reach these results using 0.34 U mL^{-1} of enzyme. Therefore, 0.68 and 1.02 U mL^{-1} of enzyme were selected for further studies.

3.4. Effect of enzymatic treatment on OS concentrations

To choose the optimal conditions is also necessary to evaluate the effect of the enzymatic treatment on COS (COSc and COSg) concentrations and GOS production to minimise the degradation of COS and the formation of GOS by transgalactosylation reactions. During enzymatic treatment, new peaks eluting between 3 and 7 min (peaks 6), were observed in both goat colostrum (Fig. 1C) and lactose control sample (Fig. 1D). Peak eluting at 3.4 min (peak 5) was identified as allolactose (6-*O*-β-D-galactosyl-D-glucose) by comparison with the GOS reference sample and with that reported elsewhere (Zolnere & Ciprovica, 2017). Moreover, an increase in 6′-galactosyl-lactose (peak 3a) was also observed. These carbohydrates were produced as a result of transgalactosylation reactions.

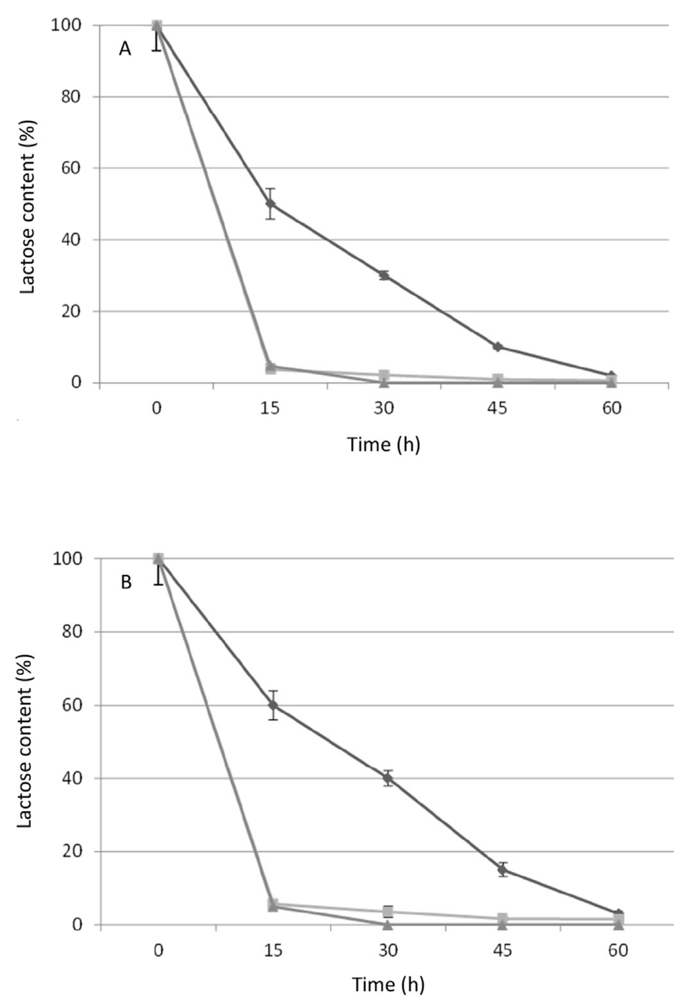

Fig. 3. Evolution of lactose content (%) during β-galactosidase treatment at short incubation times at pH 7 and using different concentrations of enzyme (◆, 0.34 U mL^{-1}; ■, 0.68 U mL^{-1}; ▲, 1.02 U mL^{-1}) in pooled goat colostrum (A) and in lactose standard solution (B).

This is in good agreement with that previously reported (Chockchaisawasdee, Athanasopoulos, Niranjan, & Rastall, 2005; Zolnere & Ciprovica, 2017) regarding the high production of GOS with linkages β-(1 → 6) after transgalactosylation reactions catalysed by Lactozym® 3000 L HP G.

Peaks eluting between 4.4 and 7 min, corresponding to GOS trisaccharides (peaks labelled as 6), were also obtained by transgalactosylation catalysed by the enzyme and were produced in both goat colostrum and lactose control. Moreover, during these treatments the production of new minor OS (t_R: 7.2 min; 11–14.5 min; 19–20 min) was detected only in goat colostrum. These carbohydrates could be the result of the hydrolysis of higher molecular weight COS during enzymatic treatment or due to transferase reactions with COSc acting as acceptors of galactose. These peaks were labelled in this work as COSh (peaks 7). In general, small qualitative differences were observed in COS profile of goat colostrum before (Fig. 1A) and after (Fig. 1C) enzymatic treatment.

Regarding quantitative analysis, Fig. 4 shows the evolution of total OS (COSc, COSg, COSh and GOS) of goat colostrum during enzymatic treatment using 0.68 U mL^{-1} (Fig. 4A) and 1.02 U mL^{-1} (Fig. 4B). Individual behaviours of all OS quantified in this study for each enzyme concentration are shown in Supplementary material Figs. S1 and S2.

GOS concentration significantly increased up to 30 min of incubation with 0.68 U mL^{-1} of enzyme and 15 min with 1.02 U mL^{-1} of enzyme, reaching the highest values under the latter conditions (70.08 mg g^{-1} colostrum sample), with allolactose experimenting the highest increase (Figs. S1 and S2). At longer incubation times, concentration of GOS decreased, probably due to their hydrolysis. In general, lower concentrations of GOS were obtained with 0.68 U mL^{-1} of enzyme (maximum: 40.12 mg g^{-1} colostrum sample at 30 min).

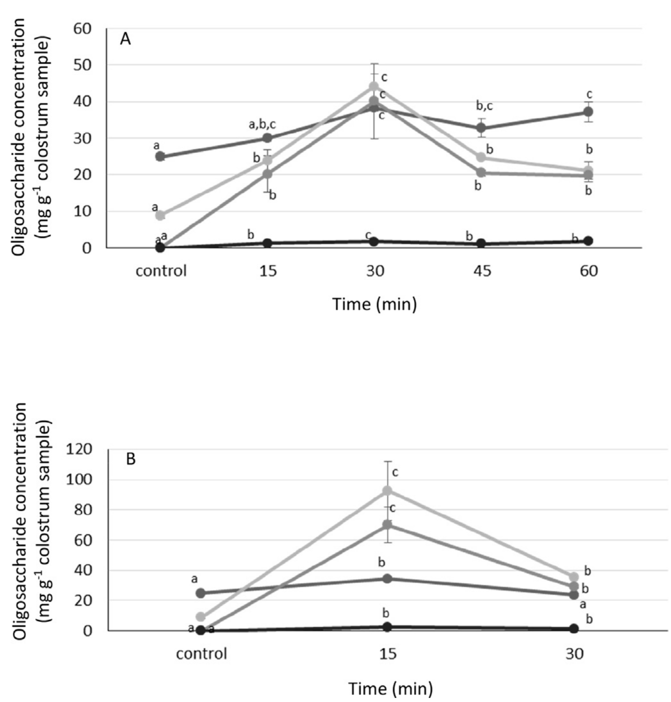

Fig. 4. Concentration (mg g^{-1}) of oligosaccharide (●, COSc; ●, COSg; ●, COSh; ●, GOS) during β-galactosidase treatment at short incubation times at pH 7 and using different concentrations of enzyme in pooled goat colostrum (A, 0.68 U mL^{-1}; B, 1.02 U mL^{-1}). Different letters indicate significant difference ($P < 0.05$) among the oligosaccharide concentrations.

Regarding COSh, a significant increase of these OS was observed for both 0.68 and 1.02 U mL^{-1} of enzyme from 15 min of incubation; however, concentrations of these carbohydrates were tiny and lower than 2.8 mg g^{-1} colostrum sample. A higher number of peaks corresponding to COSh were detected when 1.02 U mL^{-1} of enzyme was used (12 versus 7 using 1.02 U mL^{-1} and 0.68 U mL^{-1} of enzyme, respectively); however, none of them showed concentrations higher than 0.5 mg g^{-1} colostrum sample (Supplementary material Figs S1 and S2). Therefore, considering that the aim of this work was to minimise GOS production and to preserve COS profile, 0.68 U mL^{-1} of enzyme was selected as optimal concentration of β-galactosidase treatments.

A significant increase of COSg (Fig. 4A) was observed during the whole treatment, due to the production of 6'galactosyl-lactose by transgalactosylation reactions. However, the lowest concentrations of COSg were observed at 15, 45 and 60 min of incubation. Regarding COSc, no significant changes on their concentration were observed at 15 min. Therefore, 0.68 U mL^{-1} of enzyme, 37 °C, 15 min and pH 7 were selected as optimal conditions. In this sample, 24 mg COSg, 20 mg of GOS, 30 mg of COSc and 1 mg of COSh per g of pooled goat colostrum sample were present. However, high concentrations of glucose and galactose (348.5 mg g^{-1} of colostrum sample) were produced. Then, the use of an additional treatment to remove these monosaccharides using yeast was evaluated.

3.5. Yeast treatment

Pooled goat colostrum sample, previously treated with 0.68 U mL^{-1} of enzyme for 15 min was submitted to a yeast treatment (*S. cerevisiae* at 37 °C). As shown in Fig. 5, the monosaccharides glucose and galactose exhibited a sharp decrease during the first hours of treatment and only low percentages (10%) remained after 6 h. On the contrary, lactose was kept constant during the whole treatment. As expected, trehalose (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside), produced as consequence of yeast metabolism (Jules, Guillou, Francois, & Parrou, 2004), was also detected from the beginning of the treatment. This carbohydrate was totally removed at 24 h of treatment. No noticeable changes in COS were detected under these conditions; this is in good agreement with Hernández et al. (2009), who reported that carbohydrates with β-linked galactose and glucose units could not be hydrolysed by *S. cerevisiae* due to the absence of enzymes in this yeast able to hydrolyse this linkage. Therefore 24 h of *S. cerevisiae* incubation was selected as the optimum time.

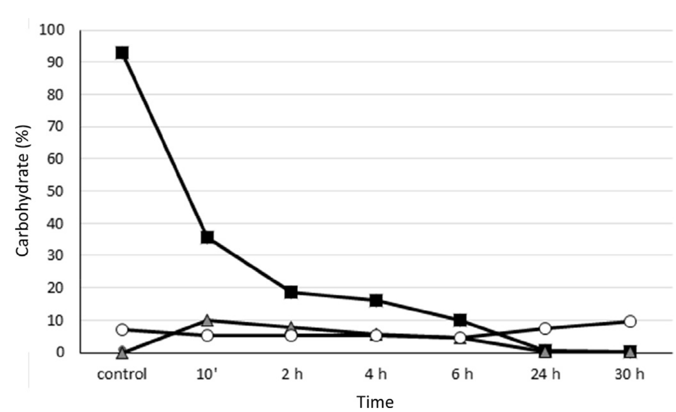

Fig. 5. Evolution of lactose (○), trehalose (▲) and monosaccharide (galactose + glucose, ■) contents (%) upon *Saccharomyces cerevisiae* incubation during 30 h at 37 °C in pooled goat colostrum previously treated with β-galactosidase from *Kluyveromyces lactis*.

4. Conclusions

Selective removal of lactose, and the resulting glucose and galactose, from pooled goat colostrum has been successfully achieved using a biotechnological procedure based on the combined use of β-galactosidase from *K. lactis* (optimal conditions: 0.68 U mL^{-1} of enzyme, 37 °C, 15 min and pH 7) and yeast from *S. cerevisiae* (optimal conditions: 37 °C, 24 h). Under these conditions 55 mg of COS, 20 mg of GOS and 21 mg of lactose per g of pooled goat colostrum sample were remaining.

This is a clean, food-grade and straightforward scalable fractionation methodology, which could be easily applied for the feasible enrichment of bioactive oligosaccharides from milk samples. The resulting high-value added product could find immediate applications in areas dealing with the development of novel nutraceuticals, functional foods and/or food supplements. The cost-efficient and food-grade conversion of goat colostrum whey permeate to manufacture novel hetero-oligosaccharides based on the combination of conveniently enriched endogenous bioactive oligosaccharides and enzymatically-synthesised GOS, but keeping the main COS structures, may be appealing for the dairy industry. This interest is sparked by the fact that the industrial production of human milk oligosaccharides is not currently feasible, which leads to the necessity of searching for suitable replacers as it is the case of GOS. Therefore, further scientific studies could be orientated towards the comparison of the functional properties of hetero-oligosaccharides obtained from different milk sources, most promisingly goat milk, to unravel their additional advantages, if any, as compared with conventional GOS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.02.012.

References

Aquino, L., de Moura Bell, J., Cohen, J. L., Liu, Y., Lee, H., de Melo Silva, V. L., et al. (2017). Purification of caprine oligosaccharides at pilot-scale. *Journal of Food Engineering, 214*, 226–235.

Barnett, A. M., Roy, N. C., McNabb, W. C., & Cookson, A. L. (2016). Effects of a semi-purified oligosaccharides-enriched fraction from caprine milk on barrier integrity and mucin production of co-culture models of the small and large intestinal epithelium. *Nutrients, 8*, 1–17.

Cardelle-Cobas, A., Fernandez, M., Salazar, N., Martinez-Villaluenga, C., Villamiel, M., Ruas-Madiedo, P., et al. (2009). Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. *Journal of Dairy Research, 76*, 317–325.

Chockchaisawasdee, S., Athanasopoulos, V. I., Niranjan, K., & Rastall, R. A. (2005). Synthesis of galacto-oligosaccharides from lactose using, β-galactosidase from *Kluyveromyces lactis*: Studies on batch and continuous UF membrane fitted bioreactors. *Biotechnology and Bioengineering, 89*, 434–444.

Daddaoua, A., Puerta, V., Requena, P., Martinez-Ferez, A., Guadix, E., de Medina, F. S., et al. (2006). Goat milk oligosaccharides are anti-inflammatory in rats with hapten-induced colitis. *Journal of Nutrition, 136*, 672–676.

de Moura Bell, J., Aquino, L., Liu, Y., Cohen, J. L., Lee, H., de Melo Silva, V. L., et al. (2016). Modeling lactose hydrolysis for efficiency and selectivity: Toward the preservation of sialyloligosaccharides in bovine colostrum whey permeate. *Journal of Dairy Science, 99*, 6157–6163.

Diez-Municio, M., Montilla, A., Moreno, F. J., & Herrero, M. (2014). A sustainable biotechnological process for the efficient synthesis of kojibiose. *Green Chemistry, 16*, 2219–2226.

FAOstat. (2018). *Food and agriculture organization of the United Nations database*. From http://faostat3.fao.org/home/E. Retrieved October 10, 2018.

Goulas, A., Tzortzis, G., & Gibson, G. R. (2007). Development of a process for the production and purification of α- and β-galactooligosaccharides from *Bifobacterium bifidum* NCIMB 41171. *International Dairy Journal, 17*, 648–656.

Hernández-Hernández, O., Calvillo, I., Lebrón-Aguilar, R., Moreno, F. J., & Sanz, M. L. (2011). Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides. *Journal of Chromatography A, 1220*, 57–67.

Hernández, O., Ruiz-Matute, A. I., Olano, A., Moreno, F. J., & Sanz, M. L. (2009). Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. *International Dairy Journal, 19*, 531–536.

Jules, M., Guillou, V., Francois, J., & Parrou, J. L. (2004). Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology, 70*, 2771–2778.

Lara-Villoslada, F., Debras, E., Nieto, A., Concha, A., Galvez, J., Lopez-Huertas, E., et al. (2006). Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. *Clinical Nutrition, 25*, 477–488.

Martin-Ortiz, A., Barile, D., Salcedo, J., Moreno, F. J., Clemente, A., Ruiz-Matute, A. I., et al. (2017). Changes in caprine milk oligosaccharides at different lactation stages analyzed by high performance liquid chromatography coupled to mass spectrometry. *Journal of Agricultural and Food Chemistry, 65*, 3523–3531.

Martin-Ortiz, A., Salcedo, J., Barile, D., Bunyatratchata, A., Moreno, F. J., Martin-Garcia, I., et al. (2016). Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry. *Journal of Chromatography A, 1428*, 143–153.

Martínez-Ferez, A., Guadix, A., & Guadix, E. M. (2006b). Recovery of caprine milk oligosaccharides with ceramic membranes. *Journal of Membrane Science, 276*, 23–30.

Martínez-Ferez, A., Guadix, A., Zapata-Montoya, J. E., & Guadix, E. M. (2008). Influence of transmembrane pressure on the separation of caprine milk oligosaccharides from protein by cross-flow ultrafiltration. *International Journal Dairy Technology, 61*, 333–339.

Martínez-Ferez, A., Rudloff, S., Guadix, A., Henkelb, C. A., Pohlentzc, G., Bozaa, J. J., et al. (2006a). Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. *International Dairy Journal, 16*, 173–181.

Martínez-Ferez, A., Zapata, J. E., Guadix, A., Almecija, M. C., Gomez, M., & Guadix, E. M. (2009). Obtention of goat milk permeates enriched in lactose-derived oligosaccharides. *Desalination, 245*, 730–736.

Martínez-Villaluenga, C., Cardelle-Cobas, A., Corzo, N., Olano, A., & Villamiel, M. (2008). Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G). *Food Chemistry, 107*, 258–264.

Mehra, R., & Kelly, P. (2006). Milk oligosaccharides: Structural and technological aspects. *International Dairy Journal, 16*, 1334–1340.

Meyrand, M., Dallas, D. C., Caillat, H., Bouvier, F., Martin, P., & Barile, D. (2013). Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize α_{S1}-casein. *Small Ruminant Research, 113*, 411–420.

Montilla, A., Megías-Pérez, R., Olano, A., & Villamiel, M. (2015). Presence of galactooligosaccharides and furosine in special dairy products designed for elderly people. *Food Chemistry, 172*, 481–485.

Nobre, C., Castro, C. C., Hantson, A., Teixeira, J. A., De Weireld, G., & Rodrigues, L. R. (2016). Strategies for the production of high-content fructo-oligosaccharides through the removal of small saccharides by co-culture or successive fermentation with yeast. *Carbohydrate Polymers, 136*, 274–281.

Oliveira, D. L., Wilbey, R. A., Grandison, A. S., Duarte, L. C., & Roseiro, L. B. (2012). Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. *International Dairy Journal, 24*, 102–106.

Pruksasri, S., & Supee, K. (2013). Sensory evaluations and stability determinations of goat milk containing galactooligosaccharides. *International Journal of Food Science and Technology, 48*, 2456–2462.

Rodriguez-Colinas, B., de Abreu, M. A., Fernandez-Arrojo, L., de Beer, R., Poveda, A., Jimenez-Barbero, J., et al. (2011). Production of galacto-oligosaccharides by the β-galactosidase from *Kluyveromyces lactis*: Comparative analysis of permeabilized cells versus soluble enzyme. *Journal of Agricultural and Food Chemistry, 59*, 10477–10484.

Rubio-Texeira, M., Arévalo-Rodríguez, M., Lequerica, J. L., & Polaina, J. (2000). Lactose utilization by *Saccharomyces cerevisiae* strains expressing *Kluveromyces lactis* LAC genes. *Journal of Biotechnology, 84*, 97–106.

Ruiz-Aceituno, L., Rodriguez-Sanchez, S., Ruiz-Matute, A. I., Ramos, L., Soria, A. C., & Sanz, M. L. (2013). Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts. *Journal of the Science of Food and Agriculture, 93*, 2797–2803.

Ruiz-Matute, A. I., Corzo-Martínez, M., Montilla, A., Olano, A., Copovi, P., & Corzo, N. (2012). Presence of mono-, di- and galactooligosaccharides in commercial lactose-free UHT dairy products. *Journal of Food Composition and Analysis, 28*, 164–169.

Ruiz-Matute, A. I., Sanz, M. L., Corzo, N., Martin-Alvarez, P. J., Ibanez, E., Martinez-Castro, I., et al. (2007). Purification of lactulose from mixtures with lactose using pressurized liquid extraction with ethanol-water at different temperatures. *Journal of Agricultural and Food Chemistry, 55*, 3346–3350.

Sanz, M. L., Gonzalez, M., de Lorenzo, C., Sanz, J., & Martinez-Castro, I. (2005). A contribution to the differentiation between nectar honey and honeydew honey. *Food Chemistry, 91*, 313–317.

Verruck, S., Dantas, A., & Prudencio, E. S. (2019). Functionality of the components from goat's milk, recent advances for functional dairy products development and its implications on human health. *Journal of Functional Foods, 52*, 243–257.

Yin, H., Bultema, J. B., Dijkhuizen, L., & van Leeuwen, S. S. (2017). Reaction kinetics and galactooligosaccharide product profiles of the β-galactosidases from *Bacillus circulans, Kluyveromyces lactis* and *Aspergillus oryzae*. *Food Chemistry, 225*, 230–238.

Yoon, S. H., Mukerjea, R., & Robyt, J. F. (2003). Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. *Carbohydrate Research, 338*, 1127–1132.

Zhu, F., Prosser, C., Zhu, Y., Otter, D., & Hemar, Y. (2018). Enzymatic formation of galactooligosaccharides in goat milk. *Food Bioscience, 26*, 38–41.

Zolnere, K., & Ciprovica, I. (2017). The comparison of commercially available β-galactosidases for dairy industry: Review. *Research of Rural Development, 1*, 215–222.

Sección 3.2: Desarrollo de un nuevo método mediante GC-MS para el análisis de oligosacáridos de leche de cabra

"Development of a gas chromatographic-mass spectrometric method for the analysis of goat milk oligosaccharides"

A. Martín-Ortiz, M.L. Sanz, F.J. Moreno, A.I. Ruiz-Matute

Development of a gas chromatographic-mass spectrometric method for the analysis of goat milk trisaccharides

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Abstract

A method for the analysis of goat milk trisaccharides by gas chromatography coupled to mass spectrometry (GC-MS) has been optimized. Different derivatization reagents (nature and amounts), temperatures and times of reaction were evaluated for several carbohydrates (i.e., 2'-fucosyl-lactose, 3'-galactosyl-lactose, 3'-sialyl-lactose, lactosamine). Accurate and reproducible results were obtained for most of the investigated carbohydrates using *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide + 1% trifluoroacetic acid at 45ºC for 30 min. Other reagents, such as trimethylsilylimidazole, were successfully used for 3'-galactosyl-lactose and lactosamine derivatization, although failed in the total derivatization of 2'-fucosyl-lactose and 3'-sialyl-lactose. Under optimal experimental conditions, linear responses (i.e., R^2 better than 0.974) were obtained in the tested range of 0.05-0.5 mg mL^{-1} of the derivatized target compounds. The developed method was successfully applied to the analysis of trisaccharides present in goat milk.

Keywords

Carbohydrate derivatization, trimethylsilyl oximes, gas chromatography-mass spectrometry, goat milk oligosaccharides

1. Introduction

Goat milk is constituted by a complex mixture of oligosaccharides with different glycosidic linkages, degrees of polymerization (DP) and monomeric composition. Among them, the existence of: i) neutral oligosaccharides, whose structures are mainly based on lactose with the addition of neutral monosaccharides such as glucose or galactose (Hex), *N*-acetylglucosamine or *N*-acetylgalactosamine (HexNAc) and fucose or deoxyhexose (Fuc) and ii) acidic oligosaccharides, containing acidic components such as *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) has been reported [1]. Goat milk is constituted by oligosaccharides with DP between 3 and 7 but mainly by DP 3. This wide structural diversity and the low abundance of these compounds in goat milk compare to that of lactose make their characterization a difficult task.

Several techniques have been proposed for the analysis of these oligosaccharides. Nuclear Magnetic Resonance (NMR) is a powerful tool for the identification of new oligosaccharides [2,3]; however it requires a high purification of target compounds [4]. Liquid chromatography coupled to mass spectrometry (LC-MS) is often the technique of choice for both qualitative and quantitative analysis, mainly using hydrophilic liquid chromatography (HILIC) as operation mode [5,6,7]. Regarding qualitative determination, it provides valuable information due to the identification capacity of mainly tandem MS analyzers, such as quadrupole-time of flight (QToF) [1,8]. However, quantitative analysis is not straightforward. Separation of isomers is usually poor [9] and a non-linear response could be obtained using QToF MS, due to the fluctuations of capillary voltage. In these cases, quantitative analysis of milk oligosaccharides is usually carried out either by high performance anion exchange chromatography

(HPAEC) with a pulse amperometric detector (PAD) [10] or by gas chromatography coupled to mass spectrometry (GC-MS) [9,11]. The problems associated to the coupling of HPAEC to MS detectors due to the extreme mobile phase conditions used and lack of individual commercial standards, hamper the proper analysis of goat milk oligosaccharides by the first technique.

Regarding GC-MS, it is one of the most widespread separation techniques for low molecular weight carbohydrates due to its high resolution, efficiency and identification capacity [12]. However, it has been mainly used for the analysis of the monomeric composition of the OS previously hydrolyzed [1] and to determine specific glycosidic linkages through methylation reactions [13]. In these cases, the qualitative and quantitative information about the individual oligosaccharide structures is lost, but the total oligosaccharide concentration is determined.

To the best of our knowledge, the analysis of intact goat milk trisaccharides by GC-MS has not been carried out yet in the literature, mainly considering the low volatility and thermal stability of these compounds and the mandatory use a previous derivatization step. Among the different established reactions, trimethylsilylation is the most widespread for the GC analysis of saccharides and polyalcohols, considering the good volatility and stability characteristics of the derivatives formed [14]. While this reaction is clearly established for hexosyl-hexoses [15,16], non-reproducible or partial derivatization reactions could take place for other carbohydrates with specific functional groups, such as imino- or acidic sugars [17]. Then, an optimization of the derivatization reaction using appropriate standards is essential previous to the analysis of real samples.

Thus, the aim of this work was to optimize a trimethylsilylation reaction (including silylation reagent, time and temperature) using different standards, both acidic and neutral, and to apply the optimal conditions to the analysis of the main goat milk carbohydrates.

2. Materials and methods

2.1. Standards, reagents and samples

Analytical standards of 2'-fucosyl-lactose, 3'-sialyl-lactose sodium salt, 6'-sialyllactose sodium salt and *N*-acetyl-lactosamine (NAL) were acquired from Carbosynth (Berkshire, UK). 3'-galactosyl-lactose, 4'-galactosyl-lactose and 6'-galactosyl-lactose were acquired from Dextra Laboratories (Reading, UK).

Goat milk sample from 30 days of lactation was obtained from one Murciano-Granadina goat from an experimental farm located at Estación Experimental del Zaidín (Granada, Spain). The sample was aliquotted and immediately frozen at −80ºC for further analysis.

All derivatization reagents such as hexamethyldisilazane (HMDS), trimethylsilylimidazole (TMSI), *N*-*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co (St. Louis, USA). TMCS were purchased from Merck (Darmstadt, Germany).

2.2. Fat, protein and lactose removal from goat milk

Fat and proteins were removed from milk using the methodology described by Martínez-Ferez et al. [18], which consist in centrifugation at 6500 × *g* for 15 min at 5ºC, kept in an ice bath for 30 min and filtrated through Whatman No. 1 filter paper to remove and discard the supernatant lipid layer. The total protein fraction was precipitated by adding two volumes of cold ethanol to the skimmed milk samples and after shaking for 2 h in an ice bath, centrifuged at 6500 × g for 30 min at 5ºC to carefully keep the supernatant. Ethanol was evaporated from the sample in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37ºC. The remaining aqueous solution containing the carbohydrate fraction was lyophilized.

Taking into account the interference in the analysis of minor oligosaccharides due to the high amounts of lactose present in goat milk, samples were submitted to size exclusion chromatography (SEC) to remove mono- and disaccharides, obtaining an enriched oligosaccharide fraction. For this purpose, 25 mL of milk carbohydrate solution (20% w/v) were injected into a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 cm × 5 cm) using water as the mobile phase at a flow of 1.5 mL min^{-1} and maintained at 4ºC. The degree of polymerization (DP) of collected fractions was determined by electrospray ionization-mass spectrometry (ESI-MS) on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector at positive polarity selecting the corresponding *m/z* values. Fractions with DP 3 were pooled and freeze-dried. Recovery of DP3 carbohydrates from SEC (n=3) was as follow: 40% galactosyl-lactoses, 45% 2'-fucosyl-lactose, 52% 3'-sialyl-lactose, 97% 6'-sialyl-lactose and 95% 6'-sialyl-lactosamine.

2.3. Derivatization procedures

For the optimization of the derivatization process, neutral (2'-fucosyl-lactose, 6'-galactosyl-lactose and *N*-acetyl-lactosamine) and acidic (3'-sialyl-lactose) carbohydrates, previously described in goat milk [7] were selected. Carbohydrates (0.2 mg mL^{-1}) were dissolved in ethanol:water (70:30, v/v), mixed with 0.1 mL of phenyl-β-D-glucoside (1 mg mL^{-1}) used as internal standard and dried under vacuum at 40 °C.

A two-step derivatization procedure was followed. The first step was based on an oximation reaction, which reduces the number of peaks corresponding to reducing sugars and simplifies the analysis of complex mixtures [16]. As previously described, oximes were formed using 350 µL of 2.5% hydroxylamine chloride in pyridine at 75ºC for 30 min [19].

Trimethylsilylation was carried out using 350 µL of the different silylation reagents [i.e., TMSI:TMCS (1:1, v/v), HMDS+10% TFA, HMDS+1% TMCS, BSTFA+1% TMCS and MSTFA+1% TMCS] at different temperatures (25, 45 and 60ºC) and times (10, 30 and 60 min). After centrifugation, 1 µL of supernatant was taken for injection. All assays were carried out in triplicate.

Goat milk oligosaccharide fraction (20 mg) were dissolved in ethanol:water (70:30, v/v), mixed with 0.1 mL of phenyl-β-D-glucoside (1 mg mL^{-1}) and dried under vacuum at 40 °C. Samples were derivatized under optimized conditions and analysed by GC.

2.4. Carbohydrate analysis

Qualitative analysis of derivatized carbohydrates was carried out by GC-MS using a 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium at ~1 mL min^{-1} as the carrier gas. A 25 m x 0.22mm, 0.10 µm film thickness fused silica column coated with HT5 (5% phenyl (equiv.) polycarborane-siloxane) from SGE (NJ, USA) was used. The oven temperature was held at 200 °C for 5 min, then programmed to 300 at 10 °C min^{-1} and held for 30 min. The injector was set at 300 °C for all the experiments and injections were made in the split mode with a split ratio 1:20. Mass spectrometer was operating in electronic impact (EI) mode at 70 eV, scanning the 40-650 *m/z* range. Interface and source temperature were 280ºC and 230ºC, respectively. Acquisition was done using a HP ChemStation software (Hewlett-Packard, Palo Alto, CA, USA).

Quantitative analysis was carried out using a HP 7890A gas chromatograph equipped with a flame ionization detector (FID) from Agilent Technologies (Palo Alto, CA, USA). Chromatographic conditions other than the carrier gas (nitrogen) and the detector temperature (300ºC) were identical to those previously described for GC-MS analysis. Carbohydrate quantitative data were obtained from FID peak areas using the internal standard procedure. Response factors (RF) relative to the internal standard were calculated over the expected concentration range of goat milk carbohydrates. The reproducibility of the method was determined on the basis of the intra- and inter-day precision for each carbohydrate standard in five separate determinations. For quantitative results, recovery of carbohydrates from SEC was considered.

3. Results and discussion

3.1. Optimization of derivatization conditions

To evaluate the effectiveness of the derivatization reaction several neutral and acidic carbohydrates with different monomeric composition (i.e. *N*-acetyl-lactosamine, 6'-galactosyl-lactose, 2'-fucosyl-lactose and 3'sialyl-lactose) were selected as representative of goat milk oligosaccharides. In all cases, a oximation step based on the use of pyridine containing 2.5% hydroxylamine hydrochloride at 75ºC for 30 min was firstly carried out. Different silylation reagents, namely, HMDS, BSTFA, TMSI and MSTFA were evaluated. It has been described that TMCS can be used in addition to other reagents to help the derivatization reaction of hindered hydroxyl groups, secondary amines, etc [20]. Then, TMCS was added at 1% in all the reactions. Moreover, and considering that TFA is also commonly used as a catalyst for the silylation of aldoses and ketoses with HMDS [12], this mixture was also considered in this study.

After evaluation of chromatographic profiles, peaks corresponding to TMS derivatives (without oximation) were not observed in any case; then, optimization of this step was not required.

Regarding silylation, Table 1 shows the yields (%) of the different carbohydrates obtained after 30 min of reaction at 45°C. In general, good silylation yields were achieved for 6’-galactosyl-lactose using all reagents, although the lowest values and the poorest reproducibility were obtained when HMDS+1%TMCS was used. In the case of *N*-acetyl-lactosamine, the best results were achieved TMSI+1%TMCS, although derivatization with MSTFA+1%TMCS was also satisfactory. On the contrary, not very good yields were obtained with BSTFA+1%TMCS and HMDS+1%TMCS.

2’-Fucosyl-lactose was successfully derivatized using MSTFA+1%TMCS, TMSI+1%TMCS and HMDS+1%TMCS, however, low yields and low reproducibility was obtained using HMDS+TFA. These results disagree with those shown by Balogh et al. [9], who proposed the use of HMDS+TFA for the analysis of 2’-fucosyl-lactose and 3’-fucosyl-lactose in human milk. To the best of our knowledge, there are not more studies published in the literature regarding the analysis of these milk carbohydrates by GC-MS.

On the other hand, good silylation yields of 3’-sialyl-lactose were obtained with HMDS+1%TFA and MSTFA+1%TMCS, nevertheless, standard deviations were high mainly with the first reagents, indicating the non-reproducibility of the analyses. Moreover, 3’-sialyl-lactose could not be determined when TMSI+1%TMCS was used as derivatization reagent.

Then, MSTFA+1%TMCS was selected as the most effective silylation reagent for the different target carbohydrates.

The temperature of the reaction was also optimized. Table 2 shows the silylation yields of the different carbohydrates at 25, 45 and 60ºC. While 25ºC were not enough to silylate the 2'-fucosyl-lactose and the 6'-galactosyl-lactose, at 45ºC, silylation was complete for all of the carbohydrates. No noticeable improvement was obtained at higher temperature (60ºC). Thus, 45ºC was selected as the optimal temperature for further reactions.

Three different silylation times (10, 30 and 60 min) were also tested (Table 3). At 10 min all carbohydrates were already derivatized, although relatively high standard deviation was obtained for 3'-sialyl-lactose. At 60 min low yields and low reproducibility were obtained for 3'-sialyl-lactose and *N*-acetyl-lactosamine. Although the aim of a derivatization reaction is to obtain a 100% yield of a single pure product, the knowledge of the reaction rate carried out under controlled conditions and the reproducibility of data are also relevant for having consistent results [20]. Then, silylation reactions using MSTFA+1%TMCS at 45ºC for 30 min were considered optimal for further studies.

3.2. Linear response and reproducibility of the method

Good linearity was obtained for calibration curves in the evaluated range (0.05–0.5 mg); correlation coefficients (R^2) ranged from 0.974 to 0.997. Intra- and inter-day reproducibilities were evaluated derivatizing 0.1 mg of neutral and acidic carbohydrate standards (n = 5) under the optimized conditions (i.e. 350 µL of MSTFA+1%TMCS at 45 ◦C for 30 min). Relative standard deviations were lower than 3%.

3.3. Application of the method to goat milk

After SEC treatment, pool goat milk sample was submitted to the optimize derivatization procedure; main goat milk oligosaccharides were correctly derivatized under selected conditions. Figure 1 shows the GC-MS profile obtained. Identification of carbohydrates was carried out comparing their retention times and mass spectra with those of the corresponding standards. Carbohydrates for which standards were not available were tentatively identified from their mass spectra. Table 4 shows the most characteristic MS fragments of each carbohydrate and their retention times. Peak 3 was identified as 2'-fucosyl-lactose, while different galactosyl lactoses were detected eluting between 16.98-17.49 min. The main peaks were assigned as 6'-galactosyl-lactose and 3'-galactosyl-lactose, which presence had been previously described in the literature [1]. Moreover, other peaks corresponding to different galactosyl-lactoses were detected. Peak 5 eluting at 17.09 min could be identified as 4'-galactosyl-lactose by comparison of its retention time and mass spectra with those of the corresponding standard. Identification of peak 4 was not possible due to the absence of standards. Regarding acidic OS, they eluted after neutral trisaccharides. Different sialyl-lactoses were detected; peaks 10 and 11 were identified as 6'-sialyl-lactose and 3'-sialyl-lactose, while peaks 12 could be tentatively identified as 6'-sialyl-lactosamine by its MS data. These carbohydrates had been previously detected by LC-MS in goat milks [1,7,8]. Peaks 8 and 9 eluted close to sialyl-lactoses (at 19.70 and 19.95 min), but showed a different mass spectra (*m/z* ion at 356, characteristic of sialyl-lactoses was not detected); then, these peaks were assigned as acidic trisaccharides although their chemical structure could not be determined.

Table 5 shows the concentration (mg L^{-1}) of the main oligosaccharides of goat milk sample obtained after SEC treatment. The most abundant carbohydrate was the 6'-

sialyl-lactose (189.73 mg L^{-1}), followed by 3'-sialyl-lactose (76.08 mg L^{-1}). Low concentrations of galactosyl-lactoses and 2'-fucosyl-lactose were also detected (31.87 and 1.14 mg L^{-1}, respectively). This concentration trend is in good agreement with data previously reported in the literature [8, 21]. It is also necessary to point out, that during SEC treatment loss of some trisaccharides is reported [22]. Then, the low concentration of galactosyl-lactoses could be attributed to this fact. However, SEC was efficient for the removal of lactose, only remaining 2.34 mg L^{-1} in the sample.

3. Conclusions

The use of MSTFA+1%TMCS as derivatization reagent at 45ºC for 30 min was found to be successful for the derivatization of acidic and neutral oligosaccharides. Trisaccharides of goat milk after SEC treatment were analyzed by GC-MS following the optimized procedure. This method can be of general application for the derivatization of OS from milks of different species before their GC analysis.

4. References

[1] Meyrand, M.; Dallas, D. C.; Caillat, H.; Bouvier, F.; Martin, P.; Barile, D., (2013) Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize αs1-casein. Small Ruminant Research, 113, 411−420.

[2] Martin-Pastor, M., Bush, C.A., (2000) Conformational studies of human milkoligosaccharides using 1H-13C one-bond NMR residual dipolar couplings. Biochemistry, 39, 4674–4683.

[3] Van Leeuwen, S.S., Schoemaker, R.J.W., Gerwig, G.J., Van Leusen-Van Kan, E.J.M., Dijkhuizen, L., Kamerling, J.P., (2014) Rapid milk group classification by 1H

NMRanalysis of Le and H epitopes in human milk oligosaccharide donor samples, Glycobiology, 24, 728–739.

[4] Chaturvedi P., Sharma, C.B., (1988) Goat milk oligosaccharides: purification and characterization by H P L C and high-field 1 H. NMR spectroscopy. Biochimica et Biophysica Acta, 967, 115-121.

[5] Ninonuevo, M.R., Park, Y., Yin, H., Zhang, J., Ward, R.E., Clowers, B.H., German, J.B., Freeman, S.L., Killeen, K., Grimm, R., Lebrilla, C.B., (2006) A strategy for annotating thehuman milk glycome, Journal of Agricultural and Food Chemistry, 54 7471–7480.

[6] Wu, S., Grimm, R., German, J.B., Lebrilla, C.B., (2011) Annotation and structural analysisof sialylated human milk oligosaccharides, Journal of Proteome Research, 10 856–868.

[7] Martín-Ortiz, A., Salcedo, J., Barile, D., Bunyatratchata, A., Moreno, F.J., Martin-Garcia, I., Clemente, A., Sanz, M.L., Ruiz-Matute, A. I. (2016). Characterization of goat milk oligosaccharides by nano-liquid chromatography on chip quadrupole time-of flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry. Journal of Chromatography A, 1428, 143-153.

[8] Martin-Ortiz, A., Barile, D., Salcedo, 374 J., Moreno, F.J., Clemente, A., Ruiz-Matute, A. I., Sanz, M.L. (2017). Changes in caprine milk oligosaccharides at different lactation stages analyzed by high performance liquid chromatography coupled to mass spectrometry. Journal of Agricultural and Food Chemistry, 65, 3523–3531.

[9] Balogh, R., Szarkab, S., Béni, S., (2015) Determination and quantification of 2’-O-fucosyllactose and 3’-O-fucosyllactose in human milk by GC–MS as O-trimethylsilyl-oxime derivatives. Journal of Pharmaceutical and Biomedical Analysis, 115, 450–456.

[10] Aquino, L., de Moura Bell, J., Cohen, J.L., Liu, Y., Lee, H., de Melo Silva, V.L., Domizio, P., Conte Junior, C.A., Barile, D., (2017) Purification of caprine oligosaccharides at pilot-scale. Journal of Food Engineering, 214, 226-235.

[11] Kunz, C., Rudloff, S., Hintelmann, A., Pohlentz, G., Egge, H., (1996) High-pHanion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides, Journal of Chromatography B: Biomedical Sciences and Applications, 685, 211–221.

[12] Ruiz-Aceituno, L., Carrero-Carralero, C., Ramos, L., Martínez-Castro, I., Sanz, M.L., (2013) Development of a carbohydrate silylation methos in ionic liquids for their gas chromatographic analysis. Analytica Chimica Acta, 787, 87-92.

[13] Viverge, D; Grimmonprez, L; Solere, M., (1997) Chemical characterization of sialyl oligosaccharides isolated from goat (Capra hircus) milk. Biochimica et Biophysica Acta-General Subjects, 1336 (2) 157-164.

[14] Ruiz-Matute, A.I., Hernández-Hernández, O., Rodríguez-Sánchez, S., Sanz, M.L., Martínez-Castro, I., (2011) Derivatization of carbohydrates for GC and GC–MS analyses. Journal of Chromatography B, 879, 1226-1240.

[15] Schummer, C., Delhomme, O., Appenzeller, B.,Wennig, R., Millet, M., (2009) Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis, Talanta, 77 (4), 1473-1482.

[16] Rojas-Escudero, E, Alarcon-Jimenez, A.L., Elizalde-Galvan, P., Rojo-Callejas, F., (2004) Optimization of carbohydrate silylation for gas chromatography. Journal of Chromatography A, 1027, 117-120.

[17] Rodríguez-Sánchez, S., Hernández, O., Ruiz-Matute, A.I., Sanz. M.L., (2011) A derivatization procedure for the simultaneous analysis of iminosugars and other low

molecular weight carbohydrates by GC-MS in mulberry (Morus sp.) Food Chemistry 126, 353-359

[18] Martinez-Ferez, A., Rudloff, S., Guadix, A., Henkel, C.A., Pohlentz, G., Boza, J.J., Guadix, E.M., Kunz, C., (2006) Goats' milk as a natural source of lactose-derived oligosaccha-rides: isolation by membrane technology, International Dairy Journal, 16, 173–181.

[19] Brokl, M., Soria, A.C., Martínez-Castro, I., Sanz, M.L., Ruiz-Matute, A.I., (2009) Characterization of O-trimethylsilyl oximes of trisaccharides by gas chromatography–mass spectrometry. Journal of Chromatography A, 1216 (46) 89-4692.

[20] Knapp D.R., (1979) Handbook of Analytical Derivatization Reactions (1st ed.), Wiley Interscience, New York

[21] Oliveira, D.L., Wilbey, R.A., Grandison, A.S., Roseiro, L.B., (2015), Milk oligosaccharides: A review. International Journal of Dairy Technology, 68 (3), 305-321.

[22] Hernández, O., Ruiz-Matute, A.I., Olano, A., Moreno, F.J., Sanz, M.L., (2009). Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. International Dairy Journal, 19 (9), 531-536.

Table 1: Silylation yields (%) of carbohydrates carried out at 45ºC 30 min using different reagents. S.D: Standard deviation in parenthesis (n=3)

	Yield (S.D)				
	HMDS+ TFA	**BSTFA+ TMCS**	**HMDS+ TMCS**	**TMSI+ TMCS**	**MSTFA+ TMCS**
2'-Fucosyl-lactose	77 (35)	135 (24)	115 (4)	114 (5)	103 (3)
3'-Sialyl-lactose	88 (39)	78 (39)	146 (53)	-	101 (18)
6'-Galactosyl-lactose	95 (14)	97 (15)	90 (17)	110 (1)	98 (1)
N-Acetyl-lactosamine	83 (13)	71 (15)	75 (16)	98 (5)	88 (6)

Table 2: Silylation yields (%) of carbohydrates using different temperature with MSTFA+1%TMCS. S.D: Standard deviation in parenthesis (n=3)

	Yield (S.D)		
	25˚C	**45˚C**	**60˚C**
2’-Fucosyl-lactose	68 (4)	101 (1)	97 (1)
3’-Sialyl-lactose	92 (2)	95 (4)	94 (1)
6’-Galactosyl-lactose	58 (3)	101 (3)	92 (6)
N-Acetyl-lactosamine	91 (1)	90 (2)	91 (2)

Table 3: Silylation yields (%) of carbohydrates using different times with MSTFA+1%TMCS. S.D: Standard deviation in parenthesis (n=3)

	Yield (S.D.)		
	10 min	**30 min**	**60 min**
2'-Fucosyl-lactose	112 (3)	102 (2)	111 (1)
3'-Sialyl-lactose	90 (17)	92 (8)	51 (22)
6'-Galactosyl-lactose	104 (6)	99 (1)	100 (1)
N-Acetyl-lactosamine	105 (1)	91 (4)	47 (14)

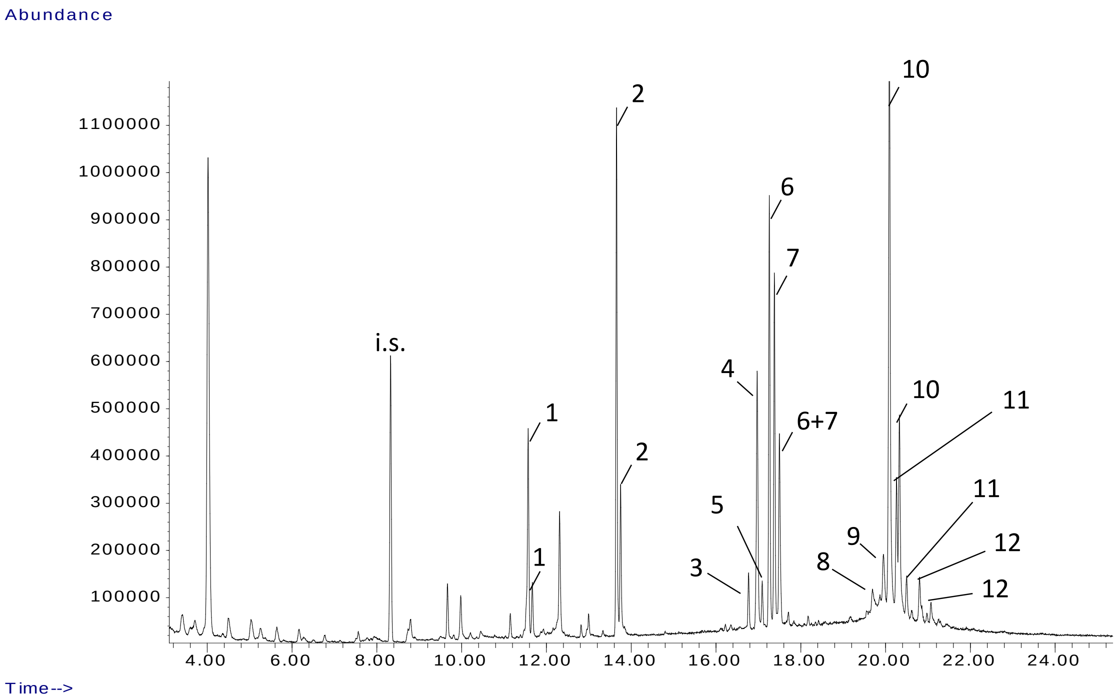
Table 4: Retention times, MS fragments and relative abundance for characteristic *m/z* ratios of TMS of goat milk oligosaccharides (OS).

Peak No.	Carbohydrate	t_R (min)	MS fragments
1	Lactose	11.57; 11.67	73 (100), 204 (65), 217 (51), 319 (21), 361 (57)
2	Lactosamine	13.65; 13.75	73 (100), 204 (51), 217 (46), 260 (34), 319 (18), 361 (53), 507 (21)
3	2'-Fucosyl-lactose	16.77	73 (100), 191 (71), 204 (127), 217 (81), 271 (30), 361 (156), 451 (8), 539 (2)
4	Galactosyl-lactose	16.98	73 (100), 204 (175), 217 (83), 319 (19), 361 (116), 451 (7), 538 (2)
5	4'-Galactosyl-lactose	17.09	73 (100), 204 (172), 217 (64), 319 (18), 361 (86), 451 (7), 538 (3)
6	6'-Galactosyl-lactose	17.26; 17.50	73 (100), 204 (188), 217 (83), 319 (23), 361 (107), 451 (10), 538 (5)
7	3'-Galactosyl-lactose	17.38; 17.49	73 (100), 204 (172), 217 (84), 319 (21), 361 (102), 451 (6), 538 (16)
8	Unknown acidic trisaccharide	19.70	73(100), 205 (53), 217 (46), 361 (12), 490 (21), 538 (10)
9	Unknown acidic trisaccharide	19.95	73(100), 205 (60), 217 (45), 361 (6), 504 (12)
10	6'-Sialyl-lactose	20.09; 20.33	73(100), 205 (82), 217 (63), 356 (13), 538 (5)
11	3'-Sialyl-lactose	20.26; 20.50	73(100), 205 (76), 217 (50), 356 (23), 538 (8)
12	Sialyl-lactosamine	20.80; 20.97	73 (100), 205 (82), 388 (18), 444 (11)

Table 5: Concentration (mg L^{-1}) of carbohydrate content of goat milk after SEC treatment.

Carbohydrate	mg L^{-1}
Lactose	2.34 (0.02)
Lactosamine	4.51 (0.05)
2’-Fucosyl-lactose	1.14 (0.08)
Galactosyl-lactose	7.19 (0.10)
4’- Galactosyl-lactose	1.17 (0.02)
6’- Galactosyl-lactose *E*	9.87 (0.02)
3’- Galactosyl-lactose *E*	8.21 (0.05)
6’+ 3’-Galactosyl-lactose *Z*	5.34 (0.04)
Sialyl-lactose	12.53 (0.52)
Sialyl-lactose	22.24 (1.2)
6’-Sialyl-lactose *E*	141.60 (5.4)
3’-Sialyl-lactose *E*	58.61 (1.7)
6’-Sialyl-lactose *Z*	48.13 (1.3)
3’-Sialyl-lactose *Z*	17.47 (0.03)
6’-Sialyl-lactosamine *E*	13.89 (0.05)
6’-Sialyl-lactosamine *Z*	1.57 (0.02)

Figure 1: Gas chromatographic profile of goat milk derivatized using MSTFA+1%TMCS at 45ºC for 30 min. 1) Lactose, 2) *N*-Acetyl-lactosamine, 3) 2’Fucosyl-lactose, 4) Galactosyl-lactose, 5) 4'-Galactosyl-lactose, 6) 6'-Galactosyl-lactose, 7) 3'-Galactosyl-lactose, 8) Unknown acidic trisaccharide, 9) Unknown acidic trisaccharide, 10) 6'-Sialyl-lactose, 11) 3'-Sialyl-lactose, 12) 6’-Sialyl-lactosamine.



Sección 3.3: Caracterización de oligosacáridos de leche de cabra mediante Nano-LC-Chip-Q-TOF MS y HILIC-MS

"Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry"

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Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry

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Nanoflow liquid chromatography–quadrupole-time of flight mass spectrometry

ABSTRACT

A detailed qualitative and quantitative characterization of goat colostrum oligosaccharides (GCO) has been carried out for the first time. Defatted and deproteinized colostrum samples, previously treated by size exclusion chromatography (SEC) to remove lactose, were analyzed by nanoflow liquid chromatography–quadrupole-time of flight mass spectrometry (Nano-LC-Chip-Q-TOF MS). Up to 78 oligosaccharides containing hexose, hexosamine, fucose, *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid monomeric units were identified in the samples, some of them detected for the first time in goat colostra. As a second step, a hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS) methodology was developed for the separation and quantitation of the main GCO, both acidic and neutral carbohydrates. Among other experimental chromatographic conditions, mobile phase additives and column temperature were evaluated in terms of retention time, resolution, peak width and symmetry of target carbohydrates. Narrow peaks (w_h: 0.2–0.6 min) and good symmetry (A_s: 0.8–1.4) were obtained for GCO using an acetonitrile:water gradient with 0.1% ammonium hydroxide at 40 °C. These conditions were selected to quantify the main oligosaccharides in goat colostrum samples. Values ranging from 140 to 315 mg L^{-1} for neutral oligosaccharides and from 83 to 251 mg L^{-1} for acidic oligosaccharides were found. The combination of both techniques resulted to be useful to achieve a comprehensive characterization of GCO.

1. Introduction

Goat milk is a complex mixture of nutritive and bioactive components with reported health benefits such as carbohydrates, lipids and proteins [1]. Although lactose is the main carbohydrate, presence of other oligosaccharides (OS) similar to those found in human milk, has been reported [2]. Among them, some studies indicate the existence of: (i) neutral oligosaccharides, whose structures are mainly based on lactose with the addition of neutral monosaccharides such as glucose or galactose (Hex), *N*-acetylglucosamine or *N*-acetylgalactosamine (Hex*N*Ac) and fucose or deoxyhexose (Fuc) and (ii) acidic oligosaccharides, containing acidic components such as *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) [3,4]. Some of these oligosaccharides, such as those containing fucosyl- or sialyl-groups have been described to have prebiotic and pathogen binding activities [5–9]. Although much effort has been focused on the composition, structure and bioactivity of OS in human milk, scarce information about both qualitative and quantitative composition of goat milk OS is available. Since it is well known that bioactive properties are directly related to OS chemical structure, the search of novel sensitive and reproducible methods for the analysis of goat milk OS is of special relevance. Moreover, it is expectable that goat colostrum has higher amounts of OS than goat milk in a similar way to bovine or human milk [3,10], representing an interesting source of bioactive OS.

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Among the different techniques used for OS analysis, high performance liquid chromatography (LC) is one of the most widespread. Human milk OS have been successfully analyzed by normal phase [11] and reverse phase LC [11–13], although a previous derivatization step is required to improve carbohydrates retention [14]. High performance anion exchange chromatography (HPAEC) provides better separation without a previous derivatization step and it has been widely used for goat milk OS characterization and quantitation [2,15–19]. However, the complex profiles obtained for OS mixtures with different linkage variants and the use of high pH and high salts concentrations in mobile phases make this technique not compatible with mass spectrometry (MS), impairing their complete characterization [20].

Hydrophilic interaction liquid chromatography (HILIC) is a powerful LC operation mode for the analysis of complex OS mixtures (galactooligosaccharides, gentiooligosaccharides, etc.), providing an appropriate resolution and good peak shapes [14,20]. Moreover, mobile phases used in HILIC are compatible with MS and even the use of a high percentage of organic solvents enhances the ionization and increase sensitivity which makes this technique appropriate for structural and glycomic research [21]. However, applications of HILIC to the analysis of mammal milks are scarce. Mariño et al. [22] developed a methodology for the analysis of bovine colostrum OS based on their fluorescent labeling, pre-fractionation by weak anionic exchange chromatography and separation by HILIC using an amide based column and a fluorescence detector. Structural assignment of 37 free glycans was carried out by a combination of HILIC analyses, exoglycosidase digestion, desalting and offline MS/MS analyses. HILIC has also been used for the successful determination of six acidic OS in bovine milk, bovine colostrum, and infant formulas [23] in combination with high-resolution selected reaction monitoring mass spectrometry (HILIC-HRSRM-MS). Nevertheless, to the best of our knowledge, HILIC-MS has not been previously used for goat milk OS analysis, being the optimization of the method a requirement for their comprehensive characterization.

In recent years, the use of nano-liquid chip-based technologies mainly coupled to MS or tandem MS (MS/MS) techniques have demonstrated to be extremely helpful for OS identification and it has been applied to milk characterization due to its high sensitivity and capacity for compositional verification [4]. Nano-LC-Chip technology coupled to time of flight (TOF) MS has been successfully used for OS analysis of human milk [24], porcine milk [25] and bovine milk [26,27]. An exhaustive characterization of OS in goat's milks with and without the genetic ability to synthesize α_{s1}-casein by nanoflow liquid chromatography–quadrupole-TOF MS (Nano-LC-Chip–Q-TOF MS) with a porous graphitized carbon column has been recently reported [4]. Twenty nine goat milk OS, 11 of which were detected by the first time, were identified and verified via MS/MS analyses. Moreover, a goat milk oligosaccharide library was also created, which gathered information available in the literature with the new identifications. This methodology has been proven to be an excellent tool for the identification of OS in mammal milks due to its high sensitivity and mass resolution; however, it has not been previously applied to the analysis of goat colostrum samples which could be of interest for further exploitation of goat colostrum oligosaccharides (GCO) as prebiotics.

In this study, goat colostrum samples, previously purified by size exclusion chromatography (SEC) to remove lactose, were firstly submitted to Nano-LC-Chip–Q-TOF MS analysis in order to exhaustively characterize their oligosaccharide fraction. As a second step, a HILIC-MS methodology was developed for the separation and quantitation of the main GCO, both acidic and neutral compounds.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade or better. Acetic acid from Normasolv (Barcelona, Spain), ammonium acetate, ammonium hydroxide from Panreac (Barcelona, Spain) and ethanol of analytical grade were purchased from Lab-Scan (Gliwice, Poland). Acetonitrile (ACN) and formic acid HPLC-MS grade were purchased from Fisher-Scientific (Fair Lawn, NJ, USA). ESI-TOF Low concentration Tuning Mix G1969-85000 was purchased from Agilent Technologies (Santa Clara, CA, USA).

Analytical standards of β-4-galactosyl-lactose, maltotriose and maltotetraose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6′-Sialyl-lactose (6′-SL) sodium salt, 3′-sialyl-lactose (3′-SL) sodium salt, 2′-fucosyl-lactose (2′-FL) and 3′-sialyl-*N*-acetyllactosamine were purchased from Carbosynth (Berkshire, UK). Standard solutions in ACN:water (50:50, v:v) were filtered through nylon FH membranes (0.22 μm; Millipore, Bedford, MA, USA) before injection.

2.2. Colostrum samples

For this study, colostrum samples from four Murciano-Granadina goats (CS1–CS4) were obtained from an experimental farm located at Estación Experimental del Zaidín (Granada, Spain). In addition, colostrum from twelve individual Murciano-Granadina goats reared at Hermanos Archiduque farm (Granada, Spain) were collected and pooled (CS5). Collected samples were immediately frozen at −80 °C until further analysis. Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line of corresponding European Directive (2010/63/EU). An experimental protocol was approved by the Ethics Committee for Animal Research from the Animal Nutrition Unit.

2.3. Fat and protein removal

Fat and proteins were removed from the samples following the methodology described by Martinez-Ferez et al. [15] with small modifications. Briefly, samples were defatted by centrifugation at 6500 × *g* for 15 min at 5 °C, then kept in an ice bath for 30 min and filtrated through Whatman No. 1 filter paper to remove the supernatant lipid layer, which was discarded.

The total protein fraction was precipitated by adding two volumes of cold ethanol to the skimmed colostrum samples and shaking for 2 h in an ice bath. The solution was then centrifuged at 6500 × *g* for 30 min at 5 °C and supernatant was carefully collected. Ethanol was evaporated from the sample in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37 °C and the remaining aqueous solution containing the carbohydrate fraction was frozen and lyophilized.

2.4. Colostrum oligosaccharides isolation

Considering the high amounts of lactose present in goat colostrum and the interference of this disaccharide in the analysis of minor oligosaccharides, samples were submitted to SEC fractionation to remove mono- and disaccharides, obtaining an enriched oligosaccharide fraction. Briefly, 25 mL of colostrum carbohydrate solution (20% wt:v) was injected into a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 cm × 5 cm) using water as the mobile phase at a flow of 1.5 mL min^{-1} and maintained at 4 °C. The degree of polymerization (DP) of collected fractions was determined by electrospray ionization-mass spectrometry (ESI-MS) on an Agilent

1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector at positive polarity selecting the corresponding m/z values. Fractions with DP ≥3 were pooled and freeze-dried.

2.5. Chromatographic analyses

2.5.1. Qualitative analysis (Nano-LC-Chip–Q-TOF MS)

Prior to MS analysis, purified and dried OS of CS1–CS5 were reconstituted to a final concentration of 0.1 mg mL^{-1} with nanopure water. MS analysis was performed with an Agilent 6520 accurate-mass Quadrupole-Time-of-Flight (Q-TOF) LC/MS with a microfluidic nano-electrospray chip (Agilent Technologies, Santa Clara, CA, USA) as previously described [28]. The chip employed contained enrichment and analytical columns, both packed with graphitized carbon. Chromatographic elution was performed with a binary gradient of 3% ACN/0.1% formic acid in water (solvent A), and 90% ACN/0.1% formic acid in water (solvent B). The column was initially equilibrated and eluted with a flow rate of 0.3 μL min^{-1} for the nano pump and 4 μL min^{-1} for the capillary pump. The 65-min gradient was programmed as follows: 0–2.5 min, 0% B; 2.5–20 min, 0–16% B; 20–30 min, 16–44% B; 30–35 min, 44–100% B; 35–45 min, 100% B; 45–65 min, 0% B. Data were acquired in the positive ionization mode with a 450–2500 mass/charge (m/z) range. The electrospray capillary voltage was 1600–1700 V. The acquisition rate was 0.63 spectra/s for both MS and MS/MS modes. Automated precursor selection was employed based on abundance, with up to 6 MS/MS per MS. The precursor isolation window was narrow (1.3 m/z). Fragmentation energy was set at 1.8 V/100 Da with an offset of −2.4 V. Internal calibration was performed using m/z 922.009 and 1221.991 as the reference masses (ESI-TOF Low concentration Tuning Mix G1969-85000, Agilent Technologies).

For OS identification, the Find Compounds by Formula function of Mass Hunter Qualitative Analysis Version B.06.00 (Agilent Technologies) was used to generate a list of deconvoluted masses selected to be in a range of 450–1500 m/z with a ≥1000 height count and a typical isotopic distribution of small biological molecules. Charge states allowed were 1–2. The function matched the masses of oligosaccharides with the goat milk oligosaccharide databases [4] creating a list of OS compositions with their specific retention time (RT).

Oligosaccharide compositions were confirmed by tandem MS (MS/MS) analysis using the same method previously described recording 6 MS/MS per each MS analysis. Compounds selected for MS/MS analysis were those with a count higher than 1000. Once OS were confirmed by MS/MS and their RT established, the relative abundance of each OS were determined by integration of individual peaks using the Batch Targeted Feature Extractor from MassHunter Profinder Version B.06.00 (Agilent Technologies) and using the MS library created in a previous work [4]. The retention time window allowed for compound – matching was ±0.5 min with the addition of ±0.25% of the RT at each time point.

2.5.2. Quantitative analysis (HILIC-Q MS)

GCO analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (5 μL) were injected using a Rheodyne 7725 valve.

LC experiments were carried out on an ethylene bridge hybrid with trifunctionally-bonded amide phase (BEH X-Bridge column); 150 mm × 4.6 mm; 3.5 μm particle size, 135 Å pore size, Waters (Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1}. Different binary gradients consisting of acetonitrile (ACN):water with addition of different additives (0.1% ammonium hydroxide, 0.1% acetic acid or 5 mM ammonium acetate) and column temperatures (30–60 °C) were assayed. Injection volume was 5 μL.

The electrospray ionization source was operated under positive or negative polarity using the following MS parameters: capillary voltage, 4 kV; temperature, 300 °C; nitrogen drying gas flow, 12 L min^{-1}; nebulizer (N_2, 99.5% purity) pressure, 276 kPa; and fragmentor voltage, 80–110 V. Adducts formed under optimal conditions were evaluated. In positive mode, mono-sodiated adducts $[M+Na]^+$ were primarily formed for the different samples and only minor abundances of $[M+K]^+$ and $[M+H]^+$ were observed. Similarly, in negative mode, $[M-H]^-$ were detected. Therefore, ions corresponding to $[M+Na]^+$ in positive mode and $[M-H]^-$ in negative mode of the oligosaccharides under analysis were monitored in SIM mode using default variable fragmentor voltages. Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Optimization of the method was carried out on the basis of RT, peak width at half height (w_h), peak tailing measured by the peak asymmetry factor (A_s): calculated as the ratio of the back half to front half widths at 10% of the peak height, and resolution (R_s), calculated as $2(t_{R2} - t_{R1})/(w_{b1} + w_{b2})$, where 1 and 2 refer to two consecutive eluting carbohydrates and w_b is the peak width at base. R_s values should be higher than 1.0 to get an appropriate separation and A_s close to 1 to get symmetric peaks.

Quantitative analysis was performed in triplicate by the external standard method, using calibration curves within the range 0.25–100 mg L^{-1} for maltotriose, maltotetraose, 2′-FL, 3′-SL and 6′-SL. Prior to quantitation of OS in all colostrum samples, matrix effect was evaluated by quantifying target analytes in solutions of CS5 before and after SEC treatment diluted in water at different ratios (1:1–1:50, v/v). Reproducibility of the method was estimated on the basis of the intra-day and inter-day precision, calculated as the relative standard deviation (RSD) of retention times and concentrations of oligosaccharide standards obtained in $n = 5$ independent measurements. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as three and ten times, respectively, the signal to noise ratio (S/N).

3. Results and discussion

3.1. Qualitative analysis of goat colostrum oligosaccharides

Several studies have pointed out the efficiency of MS related techniques to characterize OS from different biological fluids [24,25,29]. The Nano-LC-Chip–Q-TOF MS system is an excellent tool for oligosaccharide characterization in different mammal milks, allowing the identification of over 150 different OS in human milk and 55 in bovine milk [28–30]. In this work, a great variety of GCO structures were identified (based on their RT and accurate masses), showing a different profile than human or bovine milk.

Considering the high content of lactose in goat colostrum, qualitative structural analysis of GCO required a previous purification step based on SEC, leading to a reduction of 99.9% in lactose concentration (Fig. 1S in supplementary material). A partial loss of neutral OS containing three monomeric units was also observed. Table 1 presents the list of OS identified by nano-LC-QTOF MS in the five goat colostrum samples previously purified by SEC. A total of 78 compounds were identified as oligosaccharides, 59 of which have been confirmed by their MS/MS spectrum. These results indicate that GCO show greater complexity compared to those of other domestic animals [3,4,9,15,19,31]. Similar nano-LC profiles were observed among the samples analyzed (see Fig. 1), although the whole set of OS was not identified in all samples tested. Fig. 2 shows MS/MS spectrum of hexosyl-lactose, 2′-FL, and 3′-SL as representative of each OS type (neutral, fucosylated and acidic). The

Table 1
List of oligosaccharides found in goat milk colostrum samples. The empirical formula as well as neutral mass and MS/MS verification are reported for oligosaccharides in all samples.

#	Composition[a]	Formula	Neutral mass	Verified by MS/MS	Presence in individual samples and abundance[b]				
					CS1	CS2	CS3	CS4	CS5
1	1_1_1_0_0	$C_{20}H_{35}N_1O_{15}$	529.201	×	Y§	Y§	Y§	Y§	Y§
2	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.232	√	Y§	N	N	N	N
3	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.233	√	Y‡	Y‡	Y‡	Y‡	Y‡
4	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.232	√	Y¥	Y‡	Y‡	Y‡	Y‡
5	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	Y‡	Y¥	Y‡	Y‡	Y¥
6	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	Y¥	Y¥	Y¥	Y¥	Y¥
7	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.239	√	N	Y§	Y§	Y§	Y§
8	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.236	√	Y‡	N	N	N	N
9	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.239	√	N	Y§	Y§	Y§	Y§
10	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	N	Y‡	Y‡	Y§	Y‡
11	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	Y‡	Y§	Y§	Y§	Y§
12	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	Y¥	Y‡	Y‡	Y‡	Y‡
13	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	Y¥	Y‡	Y‡	Y‡	Y‡
14	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	Y‡	Y‡	Y‡	Y‡	Y‡
15	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	Y‡	Y‡	Y‡	Y‡	Y‡
16	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.296	√	Y‡	Y§	Y‡	Y‡	Y§
17	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.298	√	Y‡	Y§	Y§	Y§	Y§
18	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.296	√	Y‡	N	Y§	N	Y§
19	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.297	√	Y‡	N	N	N	N
20	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.211	√	Y¥¥	Y¥¥	Y¥¥	Y¥¥	Y¥¥
21	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.212	√	Y¥	Y¥	Y¥	Y¥	Y¥
22	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.303	×	Y‡	Y§	Y‡	Y‡	Y‡
23	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.302	√	Y‡	Y§	Y§	Y§	Y§
24	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.301	√	Y‡	Y§	Y§	Y§	Y§
25	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.302	√	Y‡	N	N	N	N
26	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.306	√	Y‡	Y‡	Y‡	Y§	Y‡
27	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.307	√	Y§	Y‡	Y‡	Y‡	Y‡
28	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.307	√	Y‡	Y‡	Y‡	Y‡	Y‡
29	2_0_1_0_0	$C_{18}H_{32}O_{15}$	488.174	√	Y§	Y§	Y§	Y‡	Y§
30	2_1_0_0_0	$C_{20}H_{35}N_{16}$	545.195	√	Y§	Y‡	Y‡	Y§	Y‡
31	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	Y‡	Y‡	Y‡	Y‡	Y‡
32	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	Y§	Y‡	Y‡	Y§	Y§
33	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	Y‡	Y‡	Y‡	Y‡	Y‡
34	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	Y‡	Y‡	Y‡	Y§	Y§
35	2_1_0_1_0	$C_{31}H_{52}N_2O_{24}$	836.293	√	Y§	Y§	Y§	Y§	Y§
36	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.276	×	Y§	N	N	N	Y§
37	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	√	Y§	Y§	Y§	Y§	Y§
38	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	×	Y‡	N	Y§	Y‡	Y§
39	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y‡	Y‡	Y‡	Y‡	Y§
40	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.168	√	Y‡	Y‡	Y‡	Y‡	Y‡
41	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y‡	Y‡	Y‡	Y‡	Y‡
42	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y‡	Y‡	Y‡	Y‡	Y‡
43	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y§	Y§	Y‡	Y‡	Y§
44	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y‡	Y‡	Y‡	Y‡	N
45	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	Y‡	Y‡	Y‡	Y‡	Y‡
46	3_0_0_0_1	$C_{29}H_{49}NO_{25}$	811.260	√	Y‡	Y§	Y‡	Y‡	Y§
47	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.266	√	Y§	Y§	Y§	Y§	Y§
48	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.264	√	Y‡	Y‡	Y‡	Y‡	Y‡
49	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.265	√	Y§	Y‡	Y‡	Y‡	Y‡
50	3_0_0_2_0	$C_{40}H_{66}N_2O_{32}$	1086.360	×	Y§	Y§	Y§	N	Y§
51	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	Y§	Y§	Y§	Y§	Y§
52	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	Y‡	Y‡	Y‡	Y‡	Y§
53	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	Y‡	Y§	Y‡	Y‡	Y§
54	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	Y§	Y§	Y§	Y§	Y§
55	3_3_0_0_0	$C_{42}H_{71}N_3O_{31}$	1113.411	×	Y§	Y§	Y§	Y§	Y§
56	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y§	Y§	Y§
57	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y‡	Y§	Y§
58	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.221	√	Y§	Y§	Y‡	Y§	Y§
59	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y§	Y‡	Y‡
60	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y§	Y‡	Y‡
61	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.220	√	Y§	Y§	Y§	Y‡	Y§
62	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.220	√	Y§	Y§	Y§	Y§	Y§
63	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y§	Y‡	Y§
64	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	Y§	Y§	Y§	Y‡	Y§
65	4_1_0_0_0	$C_{32}H_{55}NO_{26}$	869.301	√	Y‡	Y‡	Y‡	Y‡	Y‡
66	4_1_0_0_0	$C_{32}H_{55}NO_{26}$	869.302	√	Y‡	Y§	Y‡	Y‡	Y‡
67	4_2_0_0_0	$C_{40}H_{68}N_2O_{31}$	1072.385	×	Y§	Y§	Y‡	Y§	Y‡
68	4_2_0_1_0	$C_{51}H_{85}N_3O_{39}$	1363.486	×	N	N	Y§	N	Y§
69	4_2_0_1_0	$C_{51}H_{85}N_3O_{39}$	1363.481	×	Y§	Y§	Y‡	N	Y§
70	5_0_0_0_0	$C_{30}H_{52}O_{26}$	828.277	×	Y§	N	Y§	Y§	Y§
71	5_0_0_0_0	$C_{30}H_{52}O_{26}$	828.276	×	Y§	Y§	Y§	Y§	Y§
72	5_0_0_0_0	$C_{30}H_{52}O_{26}$	828.272	×	Y§	N	N	Y§	Y§
73	5_0_0_0_0	$C_{30}H_{52}O_{26}$	828.276	×	N	N	Y§	Y‡	Y§

Table 1 (*Continued*)

#	Composition[a]	Formula	Neutral mass	Verified by MS/MS	Presence in individual samples and abundance[b]				
					CS1	CS2	CS3	CS4	CS5
74	5_0_0_0_0	$C_{30}H_{52}O_{26}$	828.278	×	Y§	Y§	Y§	Y§	Y§
75	6_0_0_0_0	$C_{36}H_{62}O_{31}$	990.327	×	Y§	Y§	Y§	Y§	Y§
76	6_0_0_0_0	$C_{36}H_{62}O_{31}$	990.330	×	Y§	N	Y§	Y§	N
77	6_0_0_0_0	$C_{36}H_{62}O_{31}$	990.331	×	Y§	Y§	N	Y§	Y§
78	6_0_0_0_0	$C_{36}H_{62}O_{31}$	990.331	×	N	N	N	Y§	N

[a] Composition (in order): Hexose_HexNAc_Fucose_NeuAc_NeuGc.
[b] Relative abundance: §low abundance (<0.2%); ‡medium abundance (0.2–5%); ¥high abundance(5–20%); ¥¥very high abundance (>20%); ×compound abundance was too low to achieve good MS/MS spectra.

Table 2
Molecular ion adducts registered for colostrum goat milk oligosaccharides ($[M+Na]^+$ in positive ionization mode and $[M-H]^-$ in negative ionization mode). Symbol code: neutral (N); acidic (A).

Peak no	Oligosaccharides	$[M+Na]^+$	$[M-H]^-$	
1	α-2′-Fucosyl-lactose	511	487	N
2	Galactosyl-lactoses	527	503	N
3	3′-Sialyl-lactose and 6′-sialyl-lactose	656	632	A
4	Di-hexosyl-lactoses	689	665	N
5	Sialyl-lactosamine	697	673	A
6	Glycolyl-neuraminyl-lactosamine	713	689	A

corresponding losses of the different monomeric units of these OS are indicated in the figure. MS/MS spectra of all OS identified in the samples analyzed are also available in Fig. 2S of supplementary material.

From the 78 oligosaccharides identified, 40 (51.3%) are neutral non-fucosylated, 3 (3.8%) neutral fucosylated and 35 (44.9%) corresponded to sialylated (Ne5Ac/Neu5Gc) oligosaccharides. The predominant OS found in these colostrum fractions analyzed were sialyl-lactoses (neutral mass 633.211) followed by Hex-HexNAc-Neu5Ac (neutral mass 674.238) and Hex-HexNAc-Neu5Gc (neutral mass 690.232) residues.

Regarding neutral OS, 7 isomers of the neutral oligosaccharide galactosyl-lactose (neutral mass 504.169), 9 isomers of digalactosyl-lactose (neutral mass 666.222), 5 isomers of tri-galactosyl-lactose (neutral mass 828.277) and 4 isomers of tetragalactosyl-lactose (neutral mass 990.331) were also detected. Different isomers of *N*-acetylglucosaminyl-lactose (neutral mass 545.195) and *N*-acetylglucosaminyl-hexosyl-lactose (neutral mass 707.249) and *N*-acetylglucosaminyl-dihexosyl-lactose (neutral mass 869.301) were also found in all the colostra. These results are in good agreement with those found by Meyrand et al. [4] in goat milks, although a higher number of isomers of each oligosaccharide have been detected in the present work. Additionally, three oligosaccharides containing fucose were found in these samples (fucosyl-lactosamine, 2′-fucosyl-lactose and lacto-*N*-fucopentaose); some of these OS were detected in low abundance which hindered our ability to achieve good tandem spectra as further confirmation.

A total of 35 acidic OS, containing *N*-acetylneuraminic monomers (18), *N*-glycolylneuraminic monomers (13) and 4 containing both (isomers of sialyl-*N*-glycolyl-neuraminyl-lactose), were detected in goat colostrum (Table 1). To the best of our knowledge, these results show the greatest number of acidic OS found in goat milk, also including a higher Neu5Gc presence than that reported in previous studies (54.8% vs. 29.4%) [4,19,31].

Conventionally, a step of reduction of carbohydrate aldehydes into their alditols form is performed using sodium borohydride. However, due to the low abundance of some fucosylated OS, the reduction was not performed to avoid unwanted sample losses associated with intense washing of residual borates (incompatible with the subsequent mass spectrometry analysis). Therefore, in some cases, the oligosaccharide isomers separated by nano-LC may include α and β anomers.

3.2. Quantitative analysis of goat colostrum oligosaccharides

3.2.1. Optimization of HILIC-Q MS conditions

The pool goat colostrum CS5 sample after SEC treatment was chosen as a representative sample for the optimization of the HILIC-Q MS method. Molecular ion adducts for fucosyl-lactose, galactosyl-lactoses, sialyl-lactoses, digalactosyl-lactoses, sialyl-lactosamines and glycolyl-neuraminyl-lactosamines were selected for the optimization of HILIC-Q MS conditions at both positive and negative mode (Table 2). According to results shown in Table 1, several peaks were detected for selected m/z ions depending on the different conditions; however, only the main ones were considered for the optimization of the method. Chromatographic peaks corresponding to $[M+Na]^+$ 656 and 511 m/z ions under positive polarity and $[M-H]^-$ 632 and 487 m/z ions under negative polarity were assigned to 6′-SL and 3′-SL and 2′-FL, respectively, by comparison of their retention times and MS data with those of commercial standards.

The use of BEH-amide stationary phase was evaluated for the analysis of GCO. Different gradients of acetonitrile:water using 0.1% ammonium hydroxide as additive were assayed for the analysis of CS5 oligosaccharides. First of all, initial gradient conditions were evaluated, using different percentages of aqueous phase (10, 15 and 20%); 15% was selected for following experiments considering the appropriate retention times (14–16 min) of the first eluting compounds (acidic OS). Percentage of the aqueous phase was also increased up to 50% and 80% in 50 min; 50% was enough for the elution of target carbohydrates. Finally, gradient rate was also evaluated: aqueous phase was modified from 15% to 50% in 30, 40 and 50 min; whereas 30 and 40 min were too fast for the appropriate elution of all the compounds, 50 min provided the best conditions. These elution results were slightly improved reducing final time to 46 min, when all target carbohydrates had eluted. In all cases, 10 min were required at 50% aqueous phase to clean de column. Then, initial conditions were recovered in 1 min and finally equilibrated for 15 min. These conditions were also applied to both 0.1% acetic acid and 5 mM ammonium acetate additives.

Table 3 shows the chromatographic parameters (RT, w_h, A_s and R_s) considered for the selected carbohydrates under positive polarity. In all cases, acidic OS eluted before the neutral ones; this effect was more notable working under basic conditions (0.1% ammonium acetate as additive) where two eluting zones were clearly distinguished in CS5 profiles: (i) acidic oligosaccharides

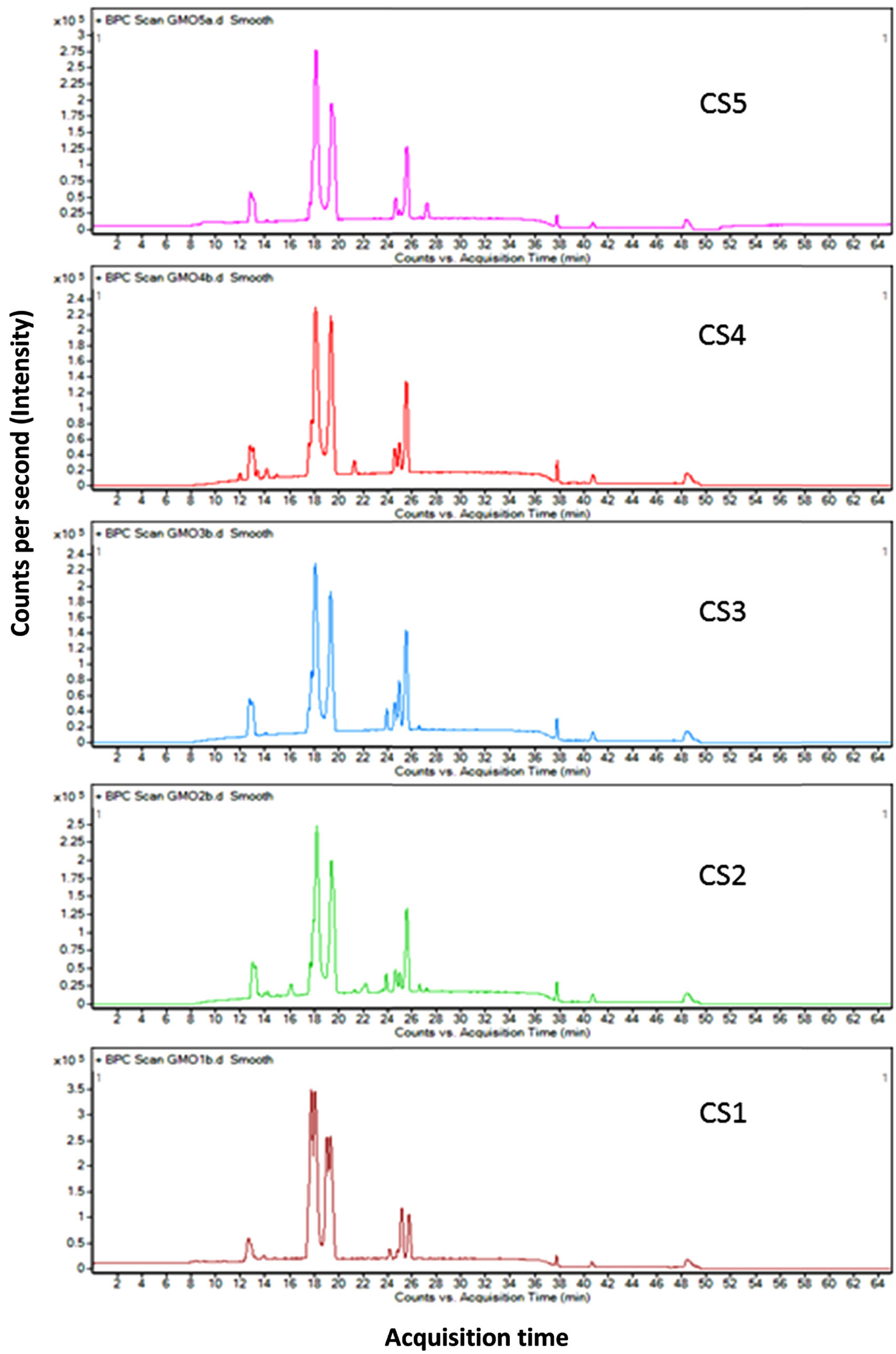

Fig. 1. Nano-LC-Chip–Q-TOF MS profiles of goat colostrum oligosaccharides: CS1, CS2, CS3, CS4, and CS5.

(14.6–16.5 min) and (ii) neutral oligosaccharides (32.6–42.5 min). The main separation mechanism in HILIC seems to be based on the partitioning between a water-enriched layer on the surface of the polar stationary phase and the relatively hydrophobic eluent [32] which mainly affect neutral carbohydrates. However, although BEH is considered a neutral stationary phase, ionization of residual surface silanol groups in this stationary phase at pH above 4 could impart negative charges to the column [32].

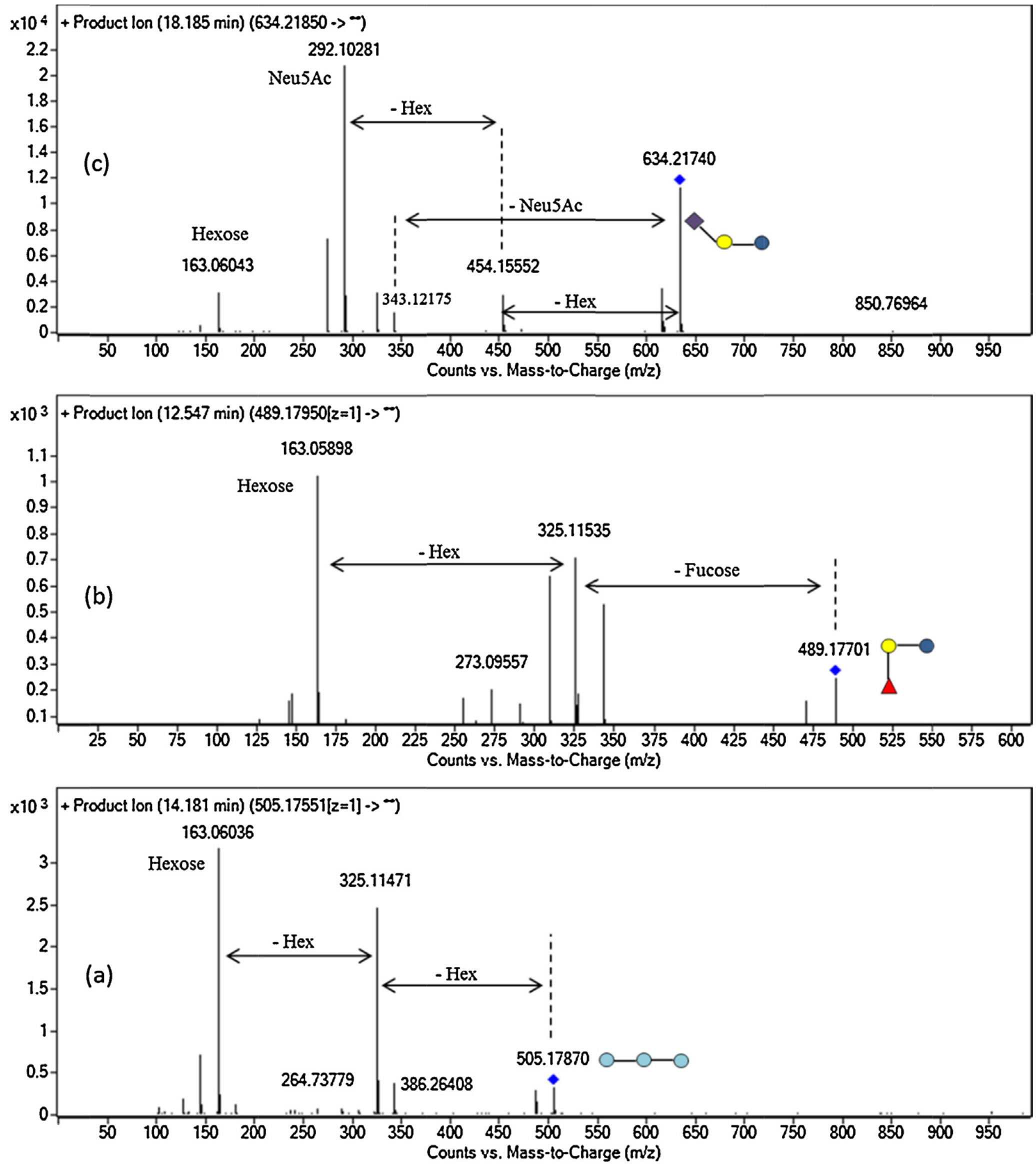

Fig. 2. MS/MS spectra of: (a) hexosyl-lactose 3_0_0_0_0 (m/z 505.176), (b) 2′-FL (m/z 489.181), and (c) 3′-SL (634.218). Light blue circle: hexose; dark blue circle: glucose; yellow circle: galactose; red triangle: fucose; violet diamond: Neu5Ac. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Chromatographic parameters obtained using different additive of aqueous phase. Acetonitrile:water elution program: 15–50% aqueous phase in 46 min, 50% aqueous phase for 10 min at 30 °C.

0.1% ammonium hydroxide ($^{w}_{s}$pH = 9.52–8.75))					0.1% acetic acid ($^{w}_{s}$pH = 4.10–3.65)					5 mM ammonium acetate ($^{w}_{s}$pH = 7.04–6.38)				
[M+Na]+	RT (min)	A_s	w_h (min)	R_s	[M+Na]+	RT (min)	A_s	w_h (min)	R_s	[M+Na]+	RT (min)	A_s	w_h (min)	R_s
656	14.6	1.3	0.3	0.2	656	22.0	2.8	0.5	0.4	713	26.4	1.1	0.3	0.1
697	14.7	1.3	0.4	0.0	713	22.3	1.8	0.5	0.1	697	26.4	0.6	0.2	0.2
713	14.7	1.0	0.5	2.5	697	22.4	0.8	0.6	2.2	656	26.5	1.0	0.2	4.1
656	16.4	1.4	0.3	0.1	656	24.4	1.2	0.5	6.4	713	28.1	1.1	0.3	0.3
713	16.5	1.4	0.3	18.5	511	28.3	0.8	0.3	6.5	656	28.2	0.9	0.3	0.7
511	32.6	1.3	0.4	3.3	527	31.1	0.7	0.6	0.6	713	28.6	0.7	0.4	4.3
527	35.6	1.2	0.6	1.9	527	31.4	1.3	0.3	1.0	511	30.8	1.1	0.2	5.8
527	36.5	1.0	0.2	1.8	527	32.1	1.1	0.4	3.6	527	33.8	0.6	0.4	1.4
689	42.5	0.7	3.6		689	41.3	1.6	2.8		527	34.5	1.1	0.2	12.2
										689	40.0	0.8	2.0	

Under working conditions, negatively charged acidic oligosaccharides (pK_a of sialic acid = 2.6) would be electrostatically repelled by the stationary phase and elute at shorter retention times.

No noticeable differences in the resolution values for the different conditions assayed were observed. Although, as previously mentioned, a better separation between acidic and neutral oligosaccharides was achieved using 0.1% ammonium hydroxide

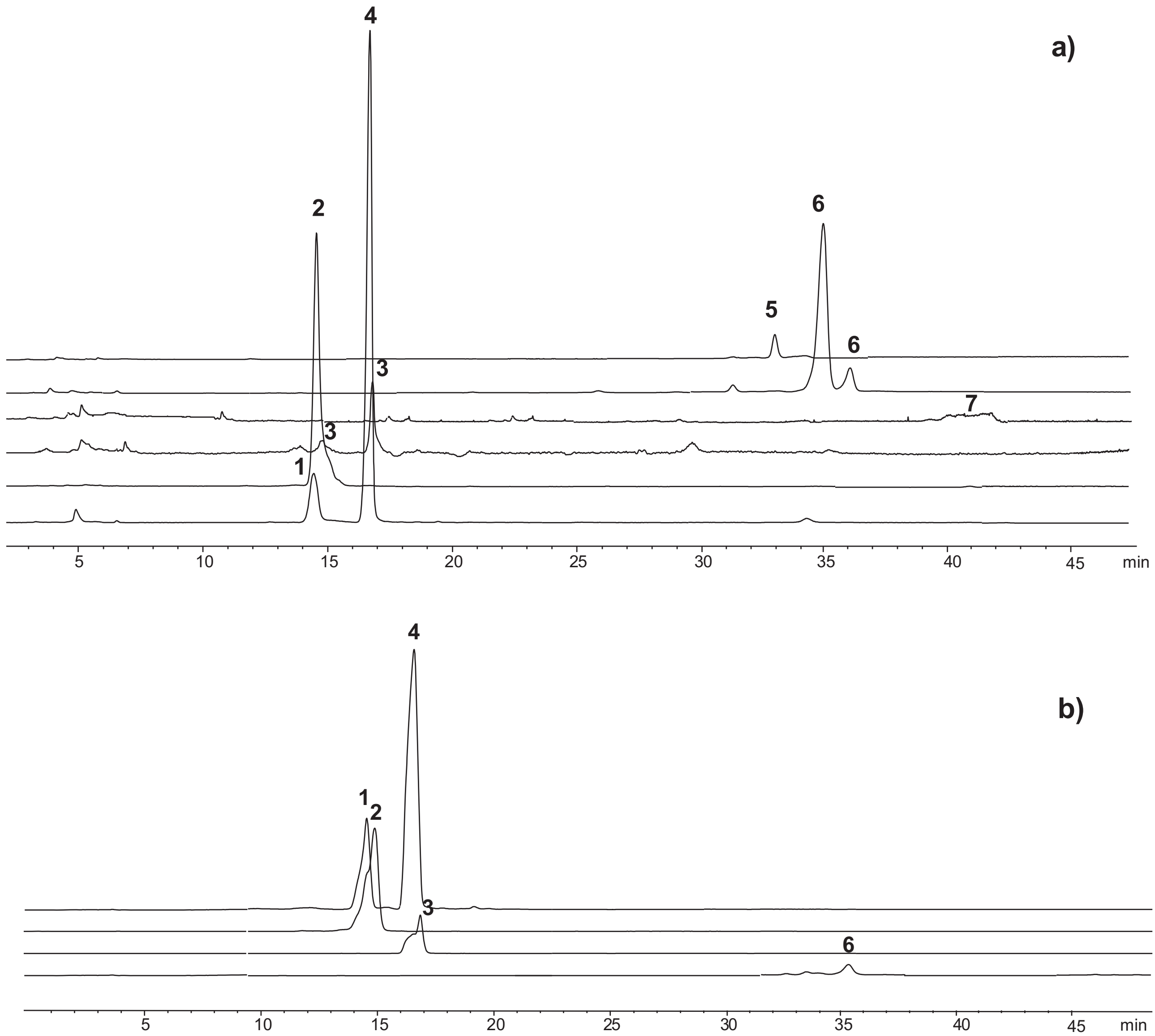

Fig. 3. Extracted ion chromatographic profile of CS5 oligosaccharides obtained using BEH amide column and 0.1% ammonium hydroxide as mobile phase additive. (a) ESI interface working under positive polarity and (b) under negative polarity. (1) 3′-Sialyl-lactose; (2) sialyl-lactosamine; (3) glycolyl-neuraminyl-lactosamine; (4) 6í-sialyl-lactose; (5) 2′-fucosyl-lactose; (6) galactosyl-lactoses; (7) di-hexosyl-lactoses.

as additive, coelutions between 3′-SL, sialyl-lactosamine and a glycolyl-neuraminyl-lactosamine and 6′-SL and a glycolyl-neuraminyl-lactosamine were observed. This behavior was also detected using 5 mM ammonium acetate as additive. A better separation was observed under acidic conditions except for 3′-SL and a glycolyl-neuraminyl-lactosamine. Different isomers of $[M+Na]^+$ 527 and 689 m/z coeluted at the same retention time under all conditions, resulting in broad peaks mainly for digalactosyl-lactoses (w_h: 2.0–3.6 min). Regarding the other oligosaccharides, in general, good peak width and symmetry values were obtained under both 0.1% ammonium hydroxide and 5 mM ammonium acetate (w_h: 0.2–0.6 min and A_s: 1.0–1.4 and w_h: 0.2–0.4 min and A_s: 0.6–1.1, respectively), whereas slightly broader peaks with poor symmetry were observed using 0.1% acetic acid as additive (w_h: 0.3–0.6 min and A_s: 0.7–2.8). Fig. 3a shows the HILIC-MS profile of registered CS5 oligosaccharides eluted under basic conditions and positive polarity.

Under negative polarity, $[M-H]^-$ ions (632, 673 and 689 m/z) corresponding to acidic carbohydrates (6′-SL and 3′-SL, sialyl-lactosamine and glycolyl-neuraminyl-lactosamine, respectively) of CS5 were clearly detected using acetonitrile:water with 0.1% acetic acid, 0.1% ammonium hydroxide and 5 mM ammonium acetate as additives. However, in all cases neutral carbohydrates could not be determined or were only slightly detected under these conditions (Fig. 3b). Therefore, further works were carried out under positive polarity.

The effect of temperature was also evaluated using BEH amide column with acetonitrile:water and 0.1% ammonium hydroxide as mobile phase. Three different temperatures (30, 40, 60 °C) were assayed (Tables 3 and 4). As expected, as temperature increased a higher decrease in RT of all the carbohydrates was observed (e.g. differences in RT values of 3.6 min for 3′-SL and 2.3 min for 2′-FL between 30 and 60 °C were observed). In general, narrower peaks with good symmetry were obtained at 40 and 60 °C (e.g. w_h = 0.2–0.4 min; A_S = 0.7–1.3), compared to elutions at 30 °C (e.g. w_h = 0.2–0.6 min; A_S = 0.7–1.4), whereas resolution was only slightly affected by temperature. Considering these results and in order to avoid high temperatures which could affect the stability

Table 4
Chromatographic parameters obtained using different oven temperatures. Acetonitrile:water + 0.1% ammonium hydroxide elution program: 15–50% aqueous phase in 46 min, 50% aqueous phase for 10 min.

40 °C					60 °C			
[M+Na]+	RT (min)	A_s	w_h (min)	R_s	RT (min)	A_s	w_h (min)	R_s
656	13.9	0.9	0.3	0.2	11.0	0.7	0.4	0.4
713	14.0	1.1	0.4	0.0	11.4	1.3	0.5	0.4
697	14.0	1.2	0.4	3.5	11.8	0.7	0.4	2.4
656	16.1	0.7	0.2	0.1	13.6	0.7	0.4	0.2
713	16.1	1.1	0.3	35.6	13.7	1.1	0.2	40.0
511	31.9	1.1	0.2	6.5	30.3	1.0	0.3	7.4
527	35.1	0.7	0.3	1.7	33.9	0.7	0.3	1.8
527	36.0	1.1	0.2	2.2	34.8	1.1	0.2	2.2
689	42.0	0.8	3.0		40.7	0.7	3.0	

of the stationary phase, 40 °C was selected for the analysis of the oligosaccharides under study.

3.2.2. Analytical parameters

Once the chromatographic conditions were selected, different analytical parameters were considered for the validation of the method before quantitative analysis.

External standard method was used for the quantitative analysis using calibration curves within the range 100–0.25 mg L^{-1} for 2′-FL, maltotriose, maltotetraose, 3′- and 6′-SL. These compounds were selected as representative of neutral fucosylated trisaccharides, neutral non-fucosylated trisaccharides, neutral tetrasaccharides and acidic OS, respectively. The obtained correlation coefficients from these calibration curves ranged from 0.92 to 0.99.

Considering that GCO are present at low levels, limit of detection (LOD), limit of quantitation (LOQ) and precision (RSD, %) data for a standard mixture including 6′-SL, 2′-FL and maltotriose, as representative standards of target compounds, were calculated. As shown in Table 5, the lowest LOD and LOQ values were obtained for 6′-SL (3.28 and 10.94 ng mL^{-1}, respectively), whereas the highest were found for 2′-FL (160.62 and 535.41 ng mL^{-1}, respectively). Good precision values were obtained for all standards analyzed (RSD ranging 6.0–8.1%).

Table 5
Limits of detection (LOD) and of quantitation (LOQ) and precision (relative standard deviation, % RSD) for a standard mixture analyzed by HILIC-Q MS using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase and 40 °C as oven temperature.

Compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Precision (RSD, %)
2′-Fucosyl-lactose	160.62	535.41	6.9
6′-Sialyl-lactose	3.28	10.94	8.1
Maltotriose	19.76	65.87	6.0
Maltotetraose	114.21	380.69	7.2

Table 6
Content (mg L^{-1}) of oligosaccharides in colostrum samples analyzed by HILIC-Q MS using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase and 40 °C as oven temperature.

	Composition[a]	[M+Na]+	RT (min)	CS1	CS2	CS3	CS4	CS5
2′-Fucosyl-lactose[c]	20100	511	32.6	2.2 (0.4)[b]	2.9 (0.1)	7.7 (0.1)	31.6 (0.3)	16 (1)
Galactosyl-lactose[c]	30000	527	35.1	145 (10)	128 (13)	125 (11)	233 (11)	266 (21)
Galactosyl-lactose[c]	30000	527	36.0	3.6 (0.5)	2.0 (0.3)	2.7 (0.7)	5 (1)	8.3 (2.2)
Sialyl-lactosamine[c]	11010	697	14.0	37.80 (0.01)	31.31 (0.01)	33 (4)	65 (6)	5.7 (0.8)
Sialyl-lactosamine[c]	11010	697	14.3	8.0 (0.6)	5.2 (0.4)	6 (2)	8.6 (0.5)	4 (1)
3′-Sialyl-lactose[c]	20010	656	13.9	3.05 (0.01)	5.73 (0.02)	12 (1)	12 (0.7)	10 (1)
6′-Sialyl-lactose[c]	20010	656	16.2	53 (5)	50 (2)	70 (1)	124 (2)	29 (4)
Glycolyl-neuraminyl-lactosamine[c]	11001	713	12.4	2.26 (0.02)	1.48 (0.01)	0.7 (0.5)	5.5 (0.5)	1.5 (0.8)
Glycolyl-neuraminyl-lactosamine[c]	11001	713	14.0	0.6 (0.1)	0.24 (0.03)	1.41 (0.02)	0.98 (0.01)	2.24 (0.03)
Glycolyl-neuraminyl-lactosamine[c]	11001	713	16.1	13.6 (0.5)	2.7 (0.1)	2.8 (0.4)	8 (1)	5.1 (0.1)
Glycolyl-neuraminyl-lactosamine[c]	11001	713	16.2	10.6 (0.6)	2.6 (0.1)	4.5 (0.7)	5 (1)	5.1 (0.1)
Glycolyl-neuraminyl-lactose[c]	20001	672	13.7	0.2 (0.1)	0.61 (0.04)	1.30 (0.01)	3.0 (0.3)	0.3 (0.5)
Glycolyl-neuraminyl-lactose[c]	20001	672	16.3	5.14 (0.03)	3.93 (0.02)	7 (1)	19 (2)	5 (1)
Glycolyl-neuraminyl-lactose[c]	20001	672	17.8	19.8 (0.3)	5.5 (0.1)	12 (1)	30 (2)	5 (2)
Fucosyl-lactosamine[c]	11100	552	30.6	3.1 (0.1)	4.1 (0.1)	6.00 (0.05)	6.15 (0.05)	5.9 (0.7)
Sialyl-hexosyl-lactose	30010	818	16.2	0.05 (0.01)	0.93 (0.02)	0.1 (0.1)	0.10 (0.01)	1.3 (0.4)
Sialyl-hexosyl-lactose	30010	818	17.4	0.05 (0.01)	–[d]	–	0.03 (0.01)	1 (1)
Sialyl-hexosyl-lactose	30010	818	17.9	0.07 (0.01)	0.08 (0.01)	–	0.05 (0.01)	1.2 (0.2)
Sialyl-glycolyl-neuraminyl-lactose	20011	963	9.5	–	–	–	–	1.1 (0.2)
Sialyl-glycolyl-neuraminyl-lactose	20011	963	12.1	0.21 (0.02)	0.27 (0.03)	0.16 (0.03)	–	1.1 (0.2)
Acetyl-glucosaminyl-hexosyl-lactose	31000	730	26.7	–	–	–	–	1.4 (0.4)
Acetyl-glucosaminyl-hexosyl-lactose	31000	730	27.6	–	0.75 (0.01)	0.4 (0.3)	1.19 (0.07)	10 (2)
Acetyl-glucosaminyl-lactose	21000	568	31.7	0.33 (0.03)	0.3 (0.1)	0.3 (0.1)	0.63 (0.01)	1.1 (0.1)
Acetyl-glucosaminyl-lactose	21000	568	32.5	0.46 (0.02)	0.26 (0.02)	0.20 (0.01)	0.7 (0.1)	3 (1)
Acetyl-glucosaminyl-lactose	21000	568	32.8	1.4 (0.2)	0.6 (0.2)	0.60 (0.01)	3.9 (0.1)	1.5 (0.6)
Acetyl-glucosaminyl-lactose	21000	568	33.9	0.11 (0.01)	0.06 (0.01)	0.29 (0.01)	0.21 (0.01)	0.7 (0.2)
Dihexosyl-lactose	40000	689	39.5	0.22 (0.01)	0.22 (0.02)	–	1.31 (0.05)	1.6 (0.1)
Dihexosyl-lactose	40000	689	40.6	–	–	–	1.98 (0.03)	2.0 (0.1)
Dihexosyl-lactose	40000	689	41.9	1.2 (0.1)	0.58 (0.01)	–	4.65 (0.05)	6.9 (0.1)
Total oligosaccharides				311.84	251.22	293.16	572.24	404.11

[a] Composition (in order): Hex_HexNAc_Fucose_NeuAc_NeuGc.
[b] Standard deviation in brackets.
[c] Oligosaccharides analyzed before SEC treatment.
[d] Non detected OS.

The potential effect on the quantitative determination of GCO of the sample matrix was also considered by analyzing different dilutions (1:1–1:50, v/v) of CS5 before and after SEC treatment. No differences in carbohydrates concentrations associated to a possible matrix effect were found for the different dilutions.

3.2.3. Quantitation of oligosaccharides in colostrum samples

Table 6 shows the quantitative data (mg L^{-1} goat colostrum) of the most abundant GCO for the five samples. Considering the potential loss of OS with three monomer units during SEC treatment, oligosaccharides marked with an asterisk in the table (2′-FL, 3′-SL, 6′-SL, sialyl-lactosamine, galactosyl-lactoses, fucosyl-lactosamine, glycolyl-neuraminyl-lactosamine and glycolyl-neuraminyl-lactose) were quantified in the original samples only after fat and protein removal (before performing SEC).

A high variability in quantified OS concentrations was observed among the different colostrum samples. CS4 showed the highest concentrations of OS (572.24 mg L^{-1}) whereas CS2 and CS3 showed the lowest values (251.22 and 293.16 mg L^{-1}, respectively). Values ranging from 140 to 315 mg L^{-1} for neutral oligosaccharides and from 83 to 251 mg L^{-1} for acidic oligosaccharides were found. The most abundant OS were galactosyl-lactoses (separation of all the isomers was not possible and these compounds were quantified together: 124.92–265.77 mg L^{-1}). Concentration of these neutral carbohydrates in colostrum samples are higher than those reported in the literature for goat milks [4]. Regarding fucosyl-oligosaccharides, 2′-FL showed higher concentrations (2.21–31.59 mg L^{-1}) than fucosyl-lactosamine (3.08–6.15 mg L^{-1}).

As indicated before, concentration of 6′-SL (28.85–123.76 mg L^{-1}) was higher than that of 3′-SL (3.05–11.99 mg L^{-1}) in all the colostrum samples. These results could suggest an OS profile closer to human milk than bovine milk, where 3′-SL is the predominant form in the latter. Relatively high amounts of sialyl-lactosamine isomers were also found in colostrum samples (5.17–8.56 and 5.66–65.12 mg L^{-1}), whereas lower concentrations of other acidic OS were observed (Table 6). Although some of these oligosaccharides have been detected in previous works in mature goat milks [4,10,15], to the best of our knowledge, this is the first time that such a large number of oligosaccharides have been quantified in goat colostrum.

4. Conclusions

A high number of both neutral and acidic OS has been detected for the first time in different goat colostrum samples by Nano-LC-Chip–Q-TOF MS. Moreover, a HILIC-Q MS method has successfully been developed for the first time for the quantitative analysis of these OS. Regarding mobile phase, acetonitrile:water with 0.1% ammonium hydroxide as additive has proven to be the most appropriate eluent to achieve good results in terms of peak width, peak symmetry and resolution. This method has proven to be successful for the quantitation of several OS in colostrum samples. Up to 0.57 g L^{-1} of total OS could be estimated, galactosyl-lactoses being the predominant carbohydrates followed by sialyl- and fucosyl-oligosaccharides, respectively. Overall, findings contained in this work strengthen the potential of goat colostrum as an efficient source of naturally-occurring bioactive OS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.09.060.

References

[1] B. Casado, M. Affolter, M. Kussmann, OMICS-rooted studies of milk proteins, oligosaccharides and lipids, J. Proteomics 73 (2009) 196–208.
[2] A. Martinez-Ferez, J.E. Zapata, A. Guadixa, M.C. Almecija, M. Gomez, E.M. Guadix, Obtention of goat milk permeates enriched in lactose-derived oligosaccharides, Desalination 245 (2009) 730–736.
[3] T. Urashima, T. Saito, T. Nakamura, M. Messer, Oligosaccharides of milk and colostrums in non-human mammals, Glycoconj. J. 18 (2001) 357–371.
[4] M. Meyrand, D.C. Dallas, H. Caillat, F. Bouvier, P. Martin, D. Barile, Comparison of milk oligosaccharides between goats with and without the genetic ability to synthesize α_{s1}-casein, Small Rumin. Res. 113 (2013) 411–420.
[5] B. Wang, J. Brand-Miller, P. McVeagh, P. Petocz, Concentration and distribution of sialic acid in human milk and infant formulas, Am. J. Clin. Nutr. 74 (2001) 510–515.
[6] Y.W. Park, Bioactive components in goat milk, in: Y.W. Park (Ed.), Bioactive Components in Milk and Dairy Products, Wiley-Blackwell, UK, 2009, pp. 43–82.
[7] C. Kunz, S. Rudloff, Health promoting aspects of milk oligosaccharides, Int. Dairy J. 16 (2006) 1341–1346.
[8] Z.-T. Yu, C. Chen, D.E. Kling, B. Liu, J.M. McCoy, M. Merighi, M. Heidtman, D.S. Newburg, The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota, Glycobiology 23 (2013) 169–177.
[9] D. Viverge, L. Grimmonprez, M. Solere, Chemical characterization of sialyl oligosaccharides isolated from goat (*Capra hircus*) milk, Biochim. Biophys. Acta 1336 (1997) 157–164.
[10] R. Mehra, P. Kelly, Milk oligosaccharides: structural and technological aspects, Int. Dairy J. 16 (2006) 1334–1340.
[11] J. Charlwood, D. Tolson, M. Dwek, P. Camilleri, A detailed analysis of neutral and acidic carbohydrates in human milk, Anal. Biochem. 273 (1999) 261–277.
[12] C. Warren, P. Chaturvedi, A. Newburg, O. Oftedal, C. Tilden, D. Newburg, Comparison of oligosaccharides in milk specimens from humans and twelve other species, Adv. Exp. Med. Biol. 501 (2001) 325–332.
[13] S. Asakuma, M. Akahori, K. Kimura, Y. Watanabe, T. Nakamura, M. Tsunemi, I. Arai, Y. Sanai, T. Urashima, Sialyl oligosaccharides of human colostrum: changes in concentration during the first three days of lactation, Biosci. Biotechnol. Biochem. 71 (2007) 1447–1451.
[14] O. Hernández-Hernández, I. Calvillo, R. Lebrón-Aguilar, F.J. Moreno, M.L. Sanz, Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides, J. Chromatogr. A 1220 (2012) 57–67.
[15] A. Martinez-Ferez, S. Rudloff, A. Guadix, C.A. Henkel, G. Pohlentz, J.J. Boza, E.M. Guadix, C. Kunz, Goats' milk as a natural source of lactose-derived oligosaccharides: isolation by membrane technology, Int. Dairy J. 16 (2006) 173–181.
[16] A. Martinez-Ferez, A. Guadix, J.E. Zapata-Montoya, E.M. Guadix, Influence of transmembrane pressure on the separation of caprine milk oligosaccharides from protein by cross-flow ultrafiltration, Int. J. Dairy Technol. 61 (2008) 333–339.
[17] F. Lara-Villoslada, E. Debras, A. Nieto, A. Concha, J. Galvez, E. Lopez-Huertas, J. Boza, C. Obled, J. Xaus, Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis, Clin. Nutr. 25 (2006) 477–488.
[18] D.L. Oliveira, R.A. Wilbey, A.S. Grandison, L.C. Duarte, L.B. Roseiro, Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation, Int. Dairy J. 24 (2012) 102–106.
[19] S. Claps, M.A. Di Napoli, L. Sepe, A.R. Caputo, D. Rufrano, A. Di Trana, G. Annicchiarico, V. Fedele, Sialyloligosaccharides content in colostrum and milk of two goat breeds, Small Rumin. Res. 121 (2014) 116–119.
[20] M. Brokl, O. Hernández-Hernández, A.C. Soria, M.L. Sanz, Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides, J. Chromatogr. A 1218 (2011) 7697–7703.
[21] M. Wuhrer, A.R. de Boer, A.M. Deelder, Structural glycomics using hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry, Mass Spectrom. Rev. 28 (2009) 192–206.
[22] K. Mariño, J.A. Lane, J.L. Abrahams, W.B. Struwe, D.J. Harvey, M. Marotta, R.M. Hickey, P.M. Rudd, Method for milk oligosaccharide profiling by 2-aminobenzamide labeling and hydrophilic interaction chromatography, Glycobiology 21 (2011) 1317–1330.
[23] B. Fong, K. Ma, P. McJarrow, Quantification of bovine milk oligosaccharides using liquid chromatography-selected reaction monitoring-mass spectrometry, J. Agric. Food Chem. 59 (2011) 9788–9795.

[24] M.R. Ninonuevo, P.D. Perkins, J. Francis, L.M. Lamotte, R.G. LoCascio, S.L. Freeman, D.A. Mills, J.B. German, R. Grimm, C.B. Lebrilla, Daily variations in oligosaccharides of human milk determined by microfluidic chips and mass spectrometry, J. Agric. Food Chem. 56 (2008) 618–626.
[25] N. Tao, K.L. Ochonicky, J.B. German, S.M. Donovan, C.B. Lebrilla, Structural determination and daily variations of porcine milk oligosaccharides, J. Agric. Food Chem. 58 (2010) 4653–4659.
[26] N. Tao, E.J. DePeters, S. Freeman, J.B. German, R. Grimm, Bovine milk glycome, J. Dairy Sci. 91 (2008) 3768–3778.
[27] D. Barile, M. Marotta, C. Chu, R. Mehra, R. Grimm, C.B. Lebrilla, J.B. German, Neutral and acidic oligosaccharides in Holstein–Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry, J. Dairy Sci. 93 (2010) 3940–3949.
[28] S. Wu, R. Grimm, J.B. German, C.B. Lebrilla, Annotation and structural analysis of sialylated human milk oligosaccharides, J. Proteome Res. 10 (2011) 856–868.
[29] D.L. Aldredge, M.R. Geronimo, S. Hua, C.C. Nwosu, C.B. Lebrilla, D. Barile, Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures, Glycobiology 23 (2013) 664–676.
[30] S. Wu, N. Tao, J.B. German, R. Grimm, C.B. Lebrilla, Development of an annotated library of neutral human milk oligosaccharides, J. Proteome Res. 9 (2010) 4138–4151.
[31] S. Albretch, J.A. Lane, K. Mariño, K.A. Al-Bushada, S.D. Carrington, R.M. Hickey, P.M. Rudd, A comparative study of free oligosaccharides in the milk of domestic animals, Br. J. Nutr. 111 (2014) 1313–1328.
[32] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC) – a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231–247.

Sección 3.4: Evolución de los oligosacáridos de la leche de cabra durante el periodo de lactación mediante HPLC-MS

"Changes in caprine milk oligosaccharides at different lactation stages analyzed by high performance liquid chromatography coupled to mass spectrometry"

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Changes in Caprine Milk Oligosaccharides at Different Lactation Stages Analyzed by High Performance Liquid Chromatography Coupled to Mass Spectrometry

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ABSTRACT: Changes of the abundance of caprine milk oligosaccharides (CMO) at different lactation stages have been evaluated by hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC−Q MS) and nanoflow liquid chromatography−quadrupole-time-of-flight mass spectrometry (nano-LC-Chip−QTOF MS). Eight major oligosaccharides (OS) were quantified at different lactation stages by HILIC−Q MS, while the use of nano-LC-Chip−QToF MS allowed expanding the study to forty-nine different OS by monitoring neutral non- and fucosylated species, as well as acidic species containing not only *N*-acetyl-neuraminic acid or *N*-glycolyl-neuraminic acid residues but also the combination of both sialic acids. Overall, the most abundant OS decreased with lactation time, whereas different trends were observed for minor OS. 6′-Sialyl-lactose was the most abundant acidic OS while galactosyl-lactose isomers were identified as the most abundant neutral OS. This is the first time that a comprehensive study regarding the changes of the abundance of CMO, both neutral and acidic, at different lactation stages is carried out.

KEYWORDS: *goat milk oligosaccharides, lactation stages, hydrophilic interaction liquid chromatography, nanoflow liquid chromatography−quadrupole-time-of-flight mass spectrometry*

■ INTRODUCTION

Human milk (HM) is considered the ideal food for neonates, not only because of its complex nutrients mixture, fulfilling infant's requirements, but also because of the presence of bioactive components, exerting important physiological effects for the neonate.[1] Among those, human milk oligosaccharides (HMOS) have been described to act as prebiotics and as potent inhibitors of bacterial adhesion to intestinal epithelial receptors.[2,3] Moreover, as they are not digested and are absorbed only in a small proportion in the gastrointestinal tract, they may play a role in the local intestinal immune system of the breast-fed infants, thus acting in the prevention of infectious diseases.[2−8]

HMOS are complex structures with different glycosidic linkages, degrees of polymerization, and monomeric composition, including hexoses (Hex, D-glucose, and D-galactose), *N*-acetyl-glucosamine (GlcNAc), L-fucose (Fuc), and sialic acid [*N*-acetyl-neuraminic acid (Neu5Ac)].[9] More than 200 different HMOS have been described to be present in HM,[10,11] with predominance of neutral fucosylated HMOS (50−70%) compared to acidic ones (10−25%).[12,13] However, due to their specific structural composition diversity, at present, it is not feasible to totally replicate the HMOS profile by synthetic methods; only HMOS of lower molecular weight have been successfully produced by molecular engineering approaches.[14] Therefore, there is great interest in finding alternative sources with similar bioactive properties to HMOS that can be exploited by the food or pharmaceutical industry on a large scale, either through direct consumption or as a functional ingredient in infant formulas, mimicking biological functions of oligosaccharides (OS) present in HM.[15,16] In this sense, naturally occurring OS isolated from milk of animal species could be considered.[17,18]

Goat milk is known to contain OS structurally similar to those present in HM[4,19−21] and in higher concentrations than milks from other farm mammals.[4] Recently, *in vitro* studies have revealed the potential prebiotic activity of OS from caprine whey.[17] Moreover, it has been proved that OS isolated from goat milk reduce intestinal inflammation in rats with induced colitis,[23] and they can improve the barrier function of epithelial cell cocultures.[24] Therefore, goat milk could be a potentially promising functional food ingredient for improving intestinal health,[24] especially if supplemented in infant formulas.

Liquid chromatography (LC) is the most widespread technique for the analysis of OS from mammal milks. Although several LC operation modes, such as normal phase (NP)[25] and

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high performance anion exchange chromatography (HPAEC),[4,14,22,23,26,27] have been widely used for the analysis of these carbohydrates, hydrophilic interaction liquid chromatography (HILIC) is considered an efficient operation mode for the analysis of complex OS mixtures,[28−31] providing appropriate resolution between isomeric OS and good peak shapes. In a recent study from our group,[20] the main OS in different goat colostra have been quantified by HILIC-Q MS. Additionally, up to 78 caprine milk oligosaccharides (CMO) structures were identified by nanoflow liquid chromatography−quadrupole-time-of-flight mass spectrometry (Nano-LC-Chip−Q-TOF MS). The combination of information provided by both techniques allowed obtaining comprehensive information, not provided in previous studies, about goat colostra OS composition.

Changes of the OS abundance of human and bovine milks at different lactation stages have been widely studied, observing a decrease in their total content during lactation and a modification in the OS profile.[28,32,33] However, little is known about the changes of CMO composition during lactation. A transcriptomic study of glycosylation-related genes in goat colostrum and milk obtained after 120 days of lactation has recently shown that most of them were more expressed in the former, which might be related to the higher OS concentration found in colostrum.[34] Recently, the content of three OS, namely 3′-sialyl-lactose (3′-SL), 6′-sialyl-lactose (6′-SL), and disialyl-lactose, in the colostra and milks of two goat breeds sampled at different stages of lactation (up to 90 days) was reported.[27,35] In general, a decrease of OS content was observed during lactation, although the 3′-SL contents increased in colostrum from 0 to 24 h. Formiga de Sousa et al.[36] also detected a decrease in sialic acid monomers, obtained after hydrolysis of CMO, at different lactation stages (85, 105, 125, and 145 days). However, to the best of our knowledge, a more comprehensive study of the evolution of both neutral and acidic CMO during lactation has not yet been carried out.

Therefore, the present work investigated the changes of the abundance of CMO during the first months of lactation in individual and pooled milks from goats of the same breed (Murciano-Granadina) by HILIC-Q MS and Nano-LC-Chip−Q-TOF MS.

■ MATERIALS AND METHODS

Chemicals and Reagents. Acetic acid was from Normasolv (Barcelona, Spain), ammonium acetate, and ammonium hydroxide were from Panreac (Barcelona, Spain), and ethanol of analytical grade was acquired from Lab-Scan (Gliwice, Poland). Acetonitrile (ACN) and formic acid (HPLC-MS grade) were obtained from Fisher-Scientific (Fair Lawn, NJ, USA). ESI-TOF Low Concentration Tuning Mix G1969-85000 was acquired from Agilent Technologies (Santa Clara, CA, USA). Nanopure water (18.2 MΩ·cm, 25 °C) was used throughout all experiments.

Analytical grade standards of 6′-sialyllactose (6′-SL) sodium salt, 3′-sialyllactose (3′-SL) sodium salt, 3′-sialyl-*N*-acetyllactosamine, 3′-galactosyl-lactose, 4′-galactosyl-lactose, and 6′-galactosyl-lactose were purchased from Carbosynth (Berkshire, UK), whereas 3α,4β-galactotriose was obtained from Dextra Laboratories (Reading, UK) and 2′-fucosyllactose (2′-FL) was from V-Laboratories (Covington, LA, USA). Nylon FH membranes (0.22 μm; Millipore, Bedford, MA, USA) were used for filtering ACN:water (50:50, v:v) standard solutions before injection.

Milk Samples. Individual milk samples from one Murciano-Granadina goat (GM) were collected at an experimental farm at Estación Experimental del Zaidín (Granada, Spain). Samples were collected at days 2, 10, 20, 30, and 40 of lactation (GM2, GM10, GM20, GM30, and GM40, respectively). Moreover, milks kindly provided by Hermanos Archiduque farm (Granada, Spain) from 12 individual Murciano-Granadina goats (GP) were collected at days 1, 7, 30, and 120 and pooled (GP1, GP7, GP30, and GP120, respectively). GM2 and GP1 samples were considered colostrum. Samples were immediately frozen at −80 °C until their analysis. The Spanish guidelines for experimental animal protection (Royal Decree 53/2013) in line with the corresponding European Directive for animal care and handling (2010/63/EU) were followed. The Ethics Committee for Animal Research from the Animal Nutrition Unit approved the experimental protocol.

Fat and Protein Removal. Samples were defatted by centrifugation at 6500*g* for 15 min at 5 °C and kept in an ice bath for 30 min. Whatman No. 1 filter paper was used for the removal of lipid layer supernatant.[4]

The removal of the protein fraction was carried out by the addition of two volumes of cold ethanol to the skimmed colostrum and milk samples. After shaking for 2 h in an ice bath, solutions were centrifuged at 6500*g* for 30 min at 5 °C. Supernatants were collected, and ethanol was removed in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37 °C. The carbohydrate fraction present in the aqueous solution was frozen and freeze-dried.

Chromatographic Analyses. *HILIC-Q MS.* CMO quantitation was performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard) with an electrospray ionization (ESI) source using the method previously optimized by Martín-Ortiz et al.[20] Samples were diluted with 50:50 (v:v) acetonitrile (ACN):water and filtered through a 0.22 μm filter (Millipore, Madrid, Spain), and 5 μL was injected.

An ethylene bridge hybrid with a trifunctionally bonded amide phase (BEH X-Bridge column; 150 × 4.6 mm; 3.5 μm particle size, 135 Å pore size, Waters, Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1} was used for LC experiments. For these assays, a gradient of ACN:water with addition of 0.1% ammonium hydroxide was used as follows: from 0 to 46 min a linear gradient from 15 to 50% aqueous phase was kept for 10 min at these conditions, and then ramped to the original composition in 1 min, and finally equilibrated for 15 min. The ESI source was operated under positive polarity using 4 kV capillary voltage at 300 °C with a nitrogen drying gas flow of 12 L min^{-1} and a nebulizer (N_2, 99.5% purity) pressure of 276 kPa; the fragmentor voltage was varied from 80 to110 V. Adducts formed under optimal conditions were evaluated. Ions corresponding to $[M + Na]^+$ of the OS under analysis were monitored in SIM mode using default variable fragmentor voltages. HPChem Station software version 10.02 (Hewlett-Packard) was used for data processing.

Quantitative analysis was performed in triplicate by the external standard method, using calibration curves within the range 5−100 mg L^{-1} for 3′-SL, 6′-SL, 3′-sialyl-*N*-acetyl-lactosamine, and 3′-galactosyl-lactose. Considering the absence of glycolyl-neuraminyl-lactose (Neu5Gc-lactose) as commercial standard, the responses of *N*-acetyl-neuraminic and *N*-glycolyl-neuraminic acids were evaluated. Negligible differences were observed between the responses of these monosaccharides; therefore, Neu5Gc-lactose isomers were quantified using 3′-SL calibration curves. The reproducibility of the method was estimated on the basis of the intraday and interday precision, calculated as the relative standard deviation (RSD) of retention times and concentrations of OS standards obtained in five independent measurements. Good precision values were obtained (RSD ranging 6−8%). The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as three and ten times, respectively, the signal to standard deviation of the noise ratio (S/σ_N). Values ranging from 0.03−0.16 mg mL^{-1} and 0.1−0.5 mg mL^{-1} were obtained for LOD and LOQ, respectively.

Nano-LC-Chip−Q-TOF MS. Sample Preparation. Considering that the high content of lactose present in goat milk would cause interference with the analysis of lower abundance OS, this disaccharide was removed from milk samples by size exclusion chromatography (SEC) using a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 × 5 cm). Water was selected as the mobile phase at a flow of 1.5 mL min^{-1} at 4 °C. The OS fraction was dissolved in water (20 wt %:v), and 25 mL was injected into the column. Fractions of 10 mL were collected. The degree of

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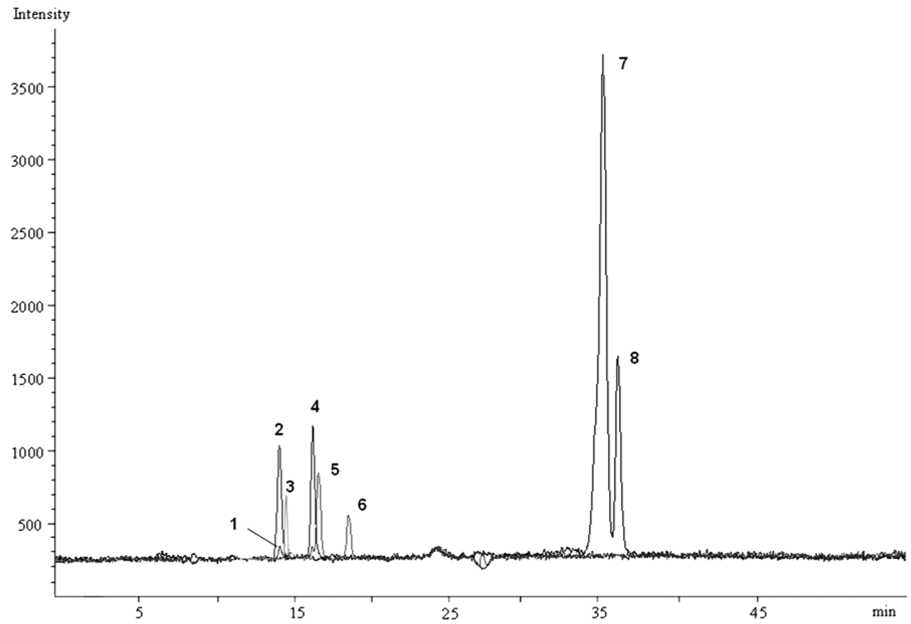

Figure 1. HILIC-Q MS profile of main oligosaccharides of GM at 20 days of lactation. See Tables 1 and 2 for peak assignation.

Table 1. Concentration (mg L^{-1}) of Oligosaccharides (OS) in Goat Milk GM Analyzed by HILIC-Q MS[a]

			Lactation period (days)				
Peak number	Carbohydrate	t_R (min)	GM2	GM10	GM20	GM30	GM40
1	Glycolyl-neuraminyl-lactose (isomer 1)	13.7	7.1 (0.3) a	–	–	–	–
2	3′-Sialyl-lactose	13.9	17.7 (3.0) a	19.4 (3.7) a	25.7 (0.8) b	12.2 (1.6) c	11.2 (1.7) c
3	Sialyl-lactosamine	14.0	28.8 (1.1) a	6.6 (1.0) b	13.3 (2.0) c	5.7 (0.2) b	–
4	6′-Sialyl-lactose	16.2	36.4 (5.1) a	26.6 (2.8) b	27.4 (1.0) b	12.6 (1.1) c	11.3 (1.3) c
5	Glycolyl-neuraminyl-lactose (isomer 2)	16.3	10.4 (1.0) a	13.2 (2.2) b	19.0 (0.1) c	6.7 (0.5) d	12.1 (0.4) a,b
6	Glycolyl-neuraminyl-lactose (isomer 3)	17.8	10.3 (1.7) a	13.6 (3.7) b	9.5 (0.1) c	4.4 (0.4) c	–
7	Galactosyl-lactose (isomer 1)	35.1	84.1 (11.0) a	43.0 (1.8) b	53.9 (2.2) c	14.9 (0.8) d	19.2 (0.8) d
8	Galactosyl-lactose (isomer 2)	36.0	4.3 (0.2) a	6.4 (1.3) b	14.0 (0.3) c	2.4 (0.3) d	5.5 (0.4) b
	Total CMO		199.1	128.8	162.8	58.9	59.3
	Acidic OS		110.7	79.4	94.9	41.6	34.6
	Neutral OS		88.4	49.4	67.9	17.3	24.7

[a]Standard deviations are presented in parentheses. Dashes (–) indicate content below LOQ (0.1–0.5 mg L^{-1}). Different letters indicate significant differences ($P < 0.05$) among the lactation stages for each OS.

polymerization (DP) of the collected fractions was determined by ESI-MS (Agilent 1200 series HPLC system, Hewlett-Packard) at positive polarity, selecting the corresponding m/z values. Fractions with DP ≥ 3 were pooled and freeze-dried.

MS Analysis. Prior to MS analysis, purified and dried OS of GM and GP samples were reconstituted to a final concentration of 0.1 mg mL^{-1} with nanopure water, with lacto-*N*-difucohexaose I (Sigma Aldrich) being added as internal standard (final concentration of 0.001g L^{-1}). MS analysis was carried out using an Agilent 6520 LC/QTOF MS with a microfluidic nanoelectrospray chip containing graphitized carbon enrichment and analytical columns (Agilent Technologies, Santa Clara, CA, USA), as previously described.[20] A binary gradient of 3% ACN/0.1% formic acid in water (solvent A) and 90% ACN/0.1% formic acid in water (solvent B) at a flow rate of 0.3 μL min^{-1} for the nano pump and an isocratic method (100% A) at a flow rate of 4 μL min^{-1} for the capillary pump were applied. Positive ionization mode with a mass/charge (m/z) range of 450–2500 was selected for data acquisition. Automated precursor selection was employed based on abundance, with up to 6 MS/MS per MS. A precursor isolation window of 1.3 m/z and a fragmentation energy set at 1.8 V/100 Da was used. Reference masses of m/z 922.009 and 1221.991 were selected for internal calibration (ESI-TOF Low concentration Tuning Mix G1969-85000, Agilent Technologies).

OS identification was performed using the Find Compounds by Formula function of Mass Hunter Qualitative Analysis Version B.06.00 (Agilent Technologies), which allowed matching the masses of OS with the caprine milk OS databases, and OS composition was confirmed by MS/MS as described in the literature.[19] The abundance of each OS relative to the internal standard (Area $_{OS}$/Area $_{i.s.}$) was obtained in triplicate.

Statistical Analysis. Statistica version 7.1 (2005) by Statsoft Inc. (Tulsa, USA) was used for statistical analyses. A one-way ANOVA test followed by a Fisher test as a post hoc comparison of means were used to determine significant differences ($P < 0.05$) in OS content among the lactation stages.

RESULTS AND DISCUSSION

To evaluate the changes in CMO abundance at different lactation stages and considering their high variability in colostra, as previously reported by Martín-Ortiz et al.,[20] two different milk collection approaches using goats of the same breed were followed in this study: (i) milks of an individual goat (GM) and (ii) pools of milk from 12 different goats (GP). GP samples could be considered as representative of milks from the Murciano-Granadina goat breed.

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Table 2. Concentrations (mg L^{-1}) of OS in the Goat Milk Pool GP Analyzed by HILIC-Q MS[a]

Peak number	Carbohydrate	t_R (min)	Lactation period (days) GP1	GP7	GP30	GP120
1	Glycolyl-neuraminyl-lactose (isomer 1)	13.7	1.7 (0.5) a,b	3.9 (1.3) c	2.8 (1.3) b,c	0.7 (0.2) a
2	3′-Sialyl-lactose	13.9	23.6 (7.2) a	9.1 (0.3) b	20.0 (2.6) a	10.2 (2.9) b
3	Sialyl-lactosamine	14.0	18.3 (1.6) a	1.9 (0.1) b	1.2 (0.1) b	0.4 (0.1) b
4	6′-Sialyl-lactose	16.2	150.7 (99.3) a	45.0 (9.6) b	86.2 (8.3) a,b	17.7 (6.7) b
5	Glycolyl-neuraminyl-lactose (isomer 2)	16.3	11.5 (4.8) a	28.5 (8.1) b	19.5 (1.5) c	11.5 (3.2) a
6	Glycolyl-neuraminyl-lactose (isomer 3)	17.8	17.0 (6.9) a,b	23.2 (4.3) a	19.6 (2.9) a	9.0 (3.9) b
7	Galactosyl-lactose (isomer 1)	35.1	250.1 (16.2) a	65.8 (14.4) b	23.1 (3.1) c	53.4 (14.1) b
8	Galactosyl-lactose (isomer 2)	36.0	15.1 (2.9) a	13.0 (4.1) a,b	5.7 (0.8) c	9.5 (2.4) b,c
	Total CMO		488	190.4	178.1	112.4
	Acidic OS		222.8	111.6	149.3	49.5
	Neutral OS		265.2	78.8	28.8	62.9

[a]Standard deviations are presented in parentheses. Different letters indicate significant differences ($P < 0.05$) among the lactation stages for each OS.

Quantitation of the Main Caprine Milk OS at Different Lactation Stages by HILIC-Q MS. Figure 1 shows the extracted ion chromatographic profile (HILIC-Q-MS) of the main OS in GM obtained at 20 days of lactation. Peaks at retention times of 13.9 and 16.2 min were assigned as 3′-SL and 6′-SL by comparison with those of commercial standards. The peak eluting at 14.0 min was identified as sialyl-lactosamine, whereas three peaks with t_R at 13.7, 16.3, and 17.8 min were assigned to different isomers of glycolyl-neuraminyl-lactose. The peak eluting at 35.1 min could be a mixture of different galactosyl-lactose isomers by comparison with the corresponding standards (3′-galactosyl-lactose, 4′-galactosyl-lactose, and 6′-galactosyl-lactose), which eluted between 34.8 and 35.4 min, whereas the peak eluting at 36.0 min could correspond to another galactosyl-lactose isomer for which a standard was not available. The presence of 3′-galactosyl-lactose in goat milks had been previously reported;[4] however, these milks can also be constituted by other different isomers of this carbohydrate.

Tables 1 and 2 show the content (mg L^{-1}) of the main OS found in GM and GP samples, respectively, at the different lactation stages. In general, the concentration of OS quantified in GP colostrum (488.0 mg L^{-1}) was higher than in GM colostrum (199.1 mg L^{-1}). Differences in OS concentration would be expected considering the variability of individual goats, as previously reported by different authors.[20,27] In both samples, the galactosyl-lactose isomer eluting at 35.1 min was the most abundant OS, followed by 6′-SL. These results agree with those found in a previous work for colostra of Murciano-Granadina goats.[20] However, Claps et al.[27] reported a higher content of 3′-SL compared to 6′-SL in colostra from two Italian goat breeds (Maltese and Garganica). These dissimilarities could be attributed to the different breeds studied. Other authors have also found significant differences in OS composition (neutral, fucosylated, and sialyl-OS) of different bovine milks depending on the breed,[37,38] reporting a higher proportion of 6′-SL in Friesian bovine colostrum and higher proportions of 3′-SL in Jersey one.

Differences in total OS concentration were observed along lactation, with higher values in colostrum and decreasing amounts during the studied period (3.3 times lower at 40 days of lactation in GM and 4.3 times lower at 120 days of lactation in GP). However, a slight increase of CMO content was observed in GM between 10 and 20 days of lactation (from 128.8 to 162.8 mg L^{-1}), a trend previously described by Tao et al.[39] in porcine milk OS.

Regarding neutral CMO, a significant decrease was observed in galactosyl-lactose isomers in GP during lactation, although the content of these OS increased from 30 to 120 days of lactation (Table 2). Nevertheless, neutral OS present in GM samples varied differently along lactation: while the major galactosyl-lactose isomer with t_R at 35.1 min decreased with time, the minor one eluting at t_R 36.0 min experienced an initial increase (colostrum to day 20), decreasing at further lactation stages (Table 1). To the best of our knowledge, there are not previous data regarding changes of neutral CMO abundance during lactation stages in the literature.

When acidic OS were evaluated, trends were dependent on the OS considered. Sialyl-lactosamine content steeply decreased during lactation in both types of samples. Although the concentration of this OS increased at 20 days in GM, it decreased below the LOQ at 40 days of lactation (Table 1), and it was present at very low levels (<2 mg L^{-1}) from the seventh day of lactation onward in GP sample (Table 2). A different behavior was observed for 3′-SL and 6′-SL concentration in both samples. Although both OS decreased during lactation, an increase was observed at day 30 in GP and at day 20 in GM samples. It is worth noting that 6′-SL showed a more noticeable decrease than 3′-SL; no significant differences between these OS contents at the end of the lactation period in both types of samples were observed. Concentrations of glycolyl-neuraminyl-lactose isomers slightly increased in GP and GM samples at early stages of lactation to further progressively decrease up to the end of the studied period (Tables 1 and 2). Furthermore, the ratio between acetyl-neuraminyl- and glycolyl-neuraminyl-OS was noticeably higher in colostrum (that is, 6.4-fold in GP1 and 3.0-fold in GM2) than in any of the samples corresponding to more advanced lactation period.

In general, sialyl-OS, which have been reported to show several physiological functions in human and other mammalian infants,[1] are more abundant in colostrum than in mature or late lactation milk. These data are in agreement with results obtained for human and bovine milks and colostra.[32,35]

Identification and Changes of Abundance of Caprine Milk OS Fraction during Lactation by Nano-LC-Chip–Q-TOF MS. To evaluate the abundance variation of minor CMO during lactation, GM and GP samples were loaded onto a SEC column to remove the high content of lactose, which could interfere in further analyses. The lactose concentration was reduced up to 99.9%, although a partial loss of trisaccharides, mainly galactosyl-lactose, was also observed in these fractions (data not shown). Considering the high sensitivity, identification

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Table 3. Evolution of OS Detected by nano-LC-QTOF MS in Murciano-Granadina Goat Milk (GM) at Different Lactation Times: Colostrum (GM2), 10 days (GM10), 20 days (GM20), 30 days (GM30), and 40 days (GM40) of Lactation[a]

						Lactation period (days)				
#	t_R	Composition[b]	Formula	Neutral Mass	Verified by MS/MS	2	10	20	30	40
1	16.50	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.232	√	+	+	−	+	+
2	18.03	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.233	√	+	+	+	+	+
3	13.85	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	+	+	+	+	+
4	16.74	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	+	−	−	−	−
5	18.34	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.239	√	+	−	−	−	−
6	16.69	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	+	++	+	+	+
7	18.04	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	+	+	+	+	+
8	22.42	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	+	+	+	+	+
9	23.75	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	+	++	+	+	+
10	25.28	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.296	√	+	+	+	+	+
11	16.99	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.211	√	+	+	+	+	+
12	23.25	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.212	√	+	+	+	+	+
13	24.92	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.303	×	+	++	+++	+	+
14	25.73	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.302	√	+	+	+	+	+
15	25.61	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.306	√	+	++	+	−	−
16	27.05	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.307	√	+	+	+	−	−
17	13.00	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	+	+	−	−	−
18	13.71	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	+	−	−	− −	− −
19	19.23	2_1_0_1_0	$C_{31}H_{52}N_2O_{24}$	836.293	√	+	+	−	+	−
20	15.49	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.276	×	+	++	+	+	+
21	17.29	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	√	+	+	++	+	+
22	19.44	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	×	+	+	+	++	+
23	9.24	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	+	+
24	11.94	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.168	√	+	+	+	−	−
25	12.71	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	+	+
26	13.39	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	+	+
27	14.37	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	+	+
28	16.35	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	++	+	−
29	24.82	3_0_0_0_1	$C_{29}H_{49}NO_{25}$	811.260	√	+	+	+	+	+
30	25.38	3_0_0_0_1	$C_{29}H_{49}NO_{25}$	811.260	√	+	+	+	+	+
31	25.23	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.266	√	+	+	+	+	+
32	25.85	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.264	√	+	+	+	+	+
33	12.26	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	+	−	−	− −	−
34	15.27	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	+	+	+	+
35	16.19	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	+	+	+	+
36	16.80	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	+	+	+	+
37	17.34	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	+	++	++	+	+
38	12.41	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+	+
39	12.87	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	++	++
40	15.93	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+	+
41	16.74	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+	+
42	20.20	3_3_0_0_0	$C_{42}H_{71}N_3O_{31}$	1113.411	×	+	+	+	+	+
43	20.62	3_3_0_0_0	$C_{42}H_{71}N_3O_{31}$	1113.411	×	+	+	+	+	+
44	13.21	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	++	+	+	+
45	16.16	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	++	−	−	−
46	19.97	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.221	√	+	+	+	+	++
47	23.75	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	+	+	+	+
48	21.92	4_2_0_0_0	$C_{40}H_{68}N_2O_{31}$	1072.385	×	+	+	++	−	+
49	26.55	4_2_0_1_0	$C_{51}H_{85}N_3O_{39}$	1363.486	×	+	−	++	−	+

[a]Symbol + indicates the presence of OS in colostrum samples. Additional + symbols indicate a significant increase ($P < 0.05$) in their relative abundances. Symbol − indicates a significant decrease ($P < 0.05$) in their relative abundances. [b]Composition (in order): Hex_Hex*N*Ac_Fucose_Neu5Ac_Neu5GC.

capacity, and small consumption of sample provided by Nano-LC-QToF MS, this technique was selected to study minor OS.

Tables 3 and 4 show the individual OS identified by nano-LC-QTOF MS in goat milks (GM and GP, respectively). The OS are cited according to their sequential monomeric composition (in order): Hex_Hex*N*Ac_Fuc_Neu5Ac_Neu5Gc, indicating the number of units that they contain. A total of 49 unique OS structures were detected. Neutral nonfucosylated OS showed the greatest diversity with 23 different structures, followed by Neu5Ac-OS with 11 structures, Neu5Gc-OS with 9, neutral

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Table 4. Evolution of OS Detected by nano-LC-QTOF MS in Murciano-Granadina Goat Milk Pool (GP) at Different Lactation Times: Colostrum (GP1), 7 days (GP7), 30 days (GP30), and 120 days (GP120)[a]

#	t_R	Composition[b]	Formula	Neutral Mass	Verified by MS/MS	Lactation period (days)			
						2	7	30	120
1	16.50	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.232	√	+	+	+	+
2	18.03	1_1_0_0_1	$C_{25}H_{42}N_2O_{20}$	690.233	√	+	+	+	+
3	13.85	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	+	+	+	+
4	16.74	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.238	√	+	−	−	−
5	18.34	1_1_0_1_0	$C_{25}H_{42}N_2O_{19}$	674.239	√	+	−	−	−
6	16.69	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	+	+++	+++	++
7	18.04	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	+	+++	+++	++
8	22.42	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.207	√	+	+	+	+
9	23.75	2_0_0_0_1	$C_{23}H_{39}NO_{20}$	649.206	√	+	+	+	+
10	25.28	2_0_0_0_2	$C_{34}H_{56}N_2O_{29}$	956.296	√	+	+	+	+
11	16.99	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.211	√	+	+	+	+
12	23.25	2_0_0_1_0	$C_{23}H_{39}NO_{19}$	633.212	√	+	+	+	+
14	25.73	2_0_0_1_1	$C_{34}H_{56}N_2O_{28}$	940.302	√	+	+	+	+
15	25.61	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.306	√	+	−	−	−
16	27.02	2_0_0_2_0	$C_{34}H_{56}N_2O_{27}$	924.307	√	+	−	+	−
17	13.00	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	+	+++	++	++
18	13.71	2_1_0_0_0	$C_{20}H_{35}NO_{16}$	545.195	√	+	++	+	+
19	19.23	2_1_0_1_0	$C_{31}H_{52}N_2O_{24}$	836.293	√	+	−	−	−
20	15.49	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.276	×	+	+	−	+
21	17.29	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	√	+	+	+	+
22	19.44	2_2_0_0_0	$C_{28}H_{48}N_2O_{21}$	748.275	×	+	−	−	+
23	9.24	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	+
24	11.94	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.168	√	+	+	−	+
25	12.71	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	− −	−	+
26	13.39	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	−	+
27	14.37	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	−	+
28	16.35	3_0_0_0_0	$C_{18}H_{32}O_{16}$	504.169	√	+	+	+	++
29	24.82	3_0_0_0_1	$C_{29}H_{49}NO_{25}$	811.260	√	+	+	+	+
30	25.38	3_0_0_0_1	$C_{29}H_{49}NO_{25}$	811.260	√	+	+	+	+
31	25.23	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.266	√	+	+	+	+
32	25.85	3_0_0_1_0	$C_{29}H_{49}NO_{24}$	795.264	√	+	+	+	+
33	12.26	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	+	+	+	+
34	15.27	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	++	++	++
35	16.19	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	+	+	+
36	16.80	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.249	√	+	+	+	+
37	17.34	3_1_0_0_0	$C_{26}H_{45}NO_{21}$	707.248	√	+	+	++	++
38	12.41	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+
39	12.87	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	++	++	+++
40	15.93	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+
41	16.74	3_1_1_0_0	$C_{32}H_{55}NO_{25}$	853.305	×	+	+	+	+
42	20.20	3_3_0_0_0	$C_{42}H_{71}N_3O_{31}$	1113.411	×	+	+	++	+++
43	20.62	3_3_0_0_0	$C_{42}H_{71}N_3O_{31}$	1113.411	×	+	+	++	+++
44	13.21	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	++	++	++
45	16.16	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	−	−	−
46	19.97	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.221	√	+	−	−	−
47	23.75	4_0_0_0_0	$C_{24}H_{42}O_{21}$	666.222	√	+	+	+	+
48	21.92	4_2_0_0_0	$C_{40}H_{68}N_2O_{31}$	1072.385	×	+	+	+	++
49	26.55	4_2_0_1_0	$C_{51}H_{85}N_3O_{39}$	1363.486	×	+	+	+++	++

[a]Symbol + indicates the presence of OS in colostrum samples. Additional + symbols indicate a significant increase ($P < 0.05$) in their relative abundances. Symbol − indicates a significant decrease ($P < 0.05$) in their relative abundances. [b]Composition (in order): Hex_Hex*N*Ac_Fucose_Neu5Ac_Neu5GC.

fucosylated OS with only 4 different structures, and Neu5Ac-Neu5Gc-OS with 2 structures. This distribution resembles bovine and porcine milk, where neutral and acidic OS show the greatest structural diversity with only a few fucosylated OS detected.[10,11,40,41]

Figure 2 shows the relative abundances (percentages) of these CMO classes in GM and GP, as well as their changes during lactation. It can be observed that, independently of the type of sample considered, the variation of OS profile during lactation is similar. By considering the OS fraction, which remained unaffected during SEC purification, the most abundant CMO

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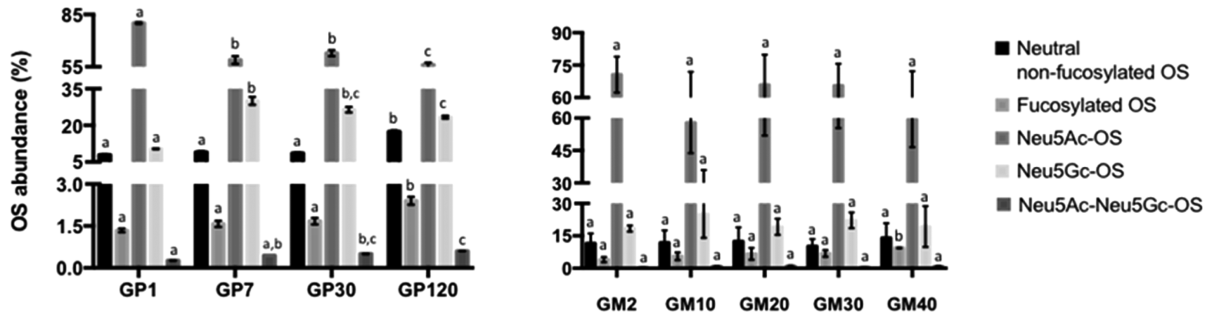

Figure 2. Relative abundance of OS classes and evolution during lactation. * Neutral OS fraction could be underestimated because the partial loss of trisaccharides during SEC purification.

present in colostrum were Neu5Ac-OS, followed by those containing Neu5Gc and neutral OS, with fucosylated OS contributing in minor proportion. As lactation advances, Neu5Ac-OS significantly decreased until the end of the period evaluated. On the contrary, the relative abundance of Neu5Gc-OS and fucosylated OS increased with lactation. A similar behavior has also been reported for fucosylated OS of porcine milk during the first week of lactation.[42]

The evolution of relative abundances of individual OS during lactation is shown in Tables 3 and 4. Differences in the evolution of neutral OS were observed not only among the different lactation stages, but also between both samples (GM and GP). Moreover, in general, different trends were observed for isomers of the same OS. In this sense, six different isomers of 3_0_0_0_0 were detected. Whereas the relative abundances of most of these isomers were kept constant during lactation in GM, a significant decrease at 7 and/or 30 days of lactation was observed in GP. Some exceptions were observed, such as the significant increase found in the isomer eluting at t_R 16.35 min at the end of lactation in GP and at 20 days in GM. In general, a similar trend in the evolution of 4_0_0_0_0 OS was observed during lactation in both milks, except for the isomer with t_R 19.97 min. This OS remained constant from GM2 to GM30 and significantly increased in GM40, whereas it decreased in GP at 7 days of lactation.

When neutral *N*-acetylated OS (mainly 2_1_0_0_0 and 3_3_0_0_0) were considered, an increase in their percentage was observed in GP with lactation, whereas these compounds either decreased or remained constant in GM. Regarding fucosylated OS (3_1_1_0_0), a significant increase of the isomer eluting at 12.87 min was observed during the studied period in both samples, whereas the other isomers remained constant.

Lower changes were observed during lactation stages on acidic OS, and more similar trends were observed in both milk samples. In general, Neu5Ac OS either continuously decreased or remained constant, with the exception of the isomer 4_2_0_1_0 (t_R 26.55 min), which experienced a significant increase at 20 or 30 days for GM or GP, respectively, and the isomer 2_0_0_2_0 (t_R 25.61 min), which significantly increased at 10 days of lactation in GM. On the contrary, most OS with Neu5Gc were constant or increased during lactation. Moreover, 2_0_0_1_1 OS (t_R 24.92 min), which contained both Neu5Ac and Neu5Gc, showed a significant increase during lactation in both samples.

It is worth noting that, although the same OS were detected in both the individual and the pool milk samples, different trends during lactation, mainly for neutral OS, were observed. Previous studies have evidenced the changes of abundance of OS of other mammal milks during lactation[20,30] and the influence of goat breed on the composition of some CMO (3′-SL, 6′SL, and disialyllactose).[24,25] However, our results reveal that CMO composition could be affected not only by the time of lactation, but also by the individual variation in goats belonging to the same breed.

In conclusion, this is the first time that a comprehensive study regarding the changes of abundance of both neutral and acidic CMO during lactation is carried out. In general, the most abundant OS decreased with lactation time, whereas different trends were observed for minor OS. Regarding acidic OS, both HILIC-Q MS and nano-LC-Chip-Q-TOF MS demonstrated that 6′-SL was the most abundant acidic OS at the first lactation stages followed by 3′-SL; however, smaller differences between these OS contents were observed at the end of the lactation period. Galactosyl-lactose isomers were the most abundant neutral OS. Milks from Murciano-Granadina goats could be considered as an attractive source of OS with potential beneficial effects for human health and could be a promising ingredient for infant formula.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Smilowitz, J. T.; Lebrilla, C. B.; Mills, D. A.; German, J. B.; Freeman, S. L. Breast milk OS: structure-function relationship in the neonate. *Annu. Rev. Nutr.* **2014**, *34*, 143−169.

DOI: 10.1021/acs.jafc.6b05104
J. Agric. Food Chem. 2017, 65, 3523−3531

(2) Kunz, C.; Rudloff, S. Health promoting aspects of milk oligosaccharides. *Int. Dairy J.* **2006**, *16*, 1341−1346.

(3) Yu, Z. T.; Chen, C.; Kling, D. E.; Liu, B.; McCoy, J. M.; Merighi, M.; Heidtman, M.; Newburg, D. S. The principal fucosylated OS of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology* **2013**, *23*, 169−177.

(4) Martinez-Ferez, A.; Rudloff, S.; Guadix, A.; Henkel, C. A.; Pohlentz, G.; Boza, J. J.; Guadix, E. M.; Kunz, C. Goats' milk as a natural source of lactose-derived OS: isolation by membrane technology. *Int. Dairy J.* **2006**, *16*, 173−181.

(5) Locascio, R. G.; Niñonuevo, M. R.; Freeman, S. L.; Sela, D. A.; Grimm, R.; Lebrilla, C. B.; Mills, D. A.; German, J. B. Glycoprofiling of bifidobacterial consumption of human milk OS demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J. Agric. Food Chem.* **2007**, *55*, 8914−8919.

(6) Bode, L. Human milk OS: Every baby needs a sugar mama. *Glycobiology* **2012**, *22*, 1147−1162.

(7) Bode, L. The functional biology of human milk oligosaccharides. *Early Hum. Dev.* **2015**, *91*, 619−622.

(8) Underwood, M. A.; Arriola, J.; Gerber, C. W.; Kaveti, A.; Kalanetra, K. M.; Kananurak, A.; Bevins, C. L.; Mills, D. A.; Dvorak, B. Bifidobacterium longum subsp infantis in experimental necrotizing enterocolitis: alterations in inflammation, innate immune response, and the microbiota. *Pediatr. Res.* **2014**, *76*, 326−333.

(9) Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. OS in human milk: structural, functional, and metabolic aspects. *Annu. Rev. Nutr.* **2000**, *20*, 699−722.

(10) Wu, S.; Tao, N.; German, J. B.; Grimm, R.; Lebrilla, C. B. Development of an annotated library of neutral human milk oligosaccharides. *J. Proteome Res.* **2010**, *9*, 4138−4151.

(11) Wu, S.; Grimm, R.; German, J. B.; Lebrilla, C. B. Annotation and structural analysis of sialylated human milk oligosaccharides. *J. Proteome Res.* **2011**, *10*, 856−868.

(12) Niñonuevo, M. R.; Bode, L. Infant formula OS opening the gates. *Pediatr. Res.* **2008**, *64*, 8−10.

(13) Kobata, A. Structures, classification and biosynthesis of human milk OS. In *Prebiotics and Probiotics in Human Milk*; McGuire, M., McGuire, M., Bode, L., Eds.; Elsevier: London, UK, 2016; pp 17−44.

(14) Goehring, K. C.; Marriage, B. J.; Oliver, J. S.; Wilder, J. A.; Barrett, E. G.; Buck, R. H. Similar to those who are breasted, infants fed a formula containing 2′-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. *J. Nutr.* **2016**, *146*, 2559−2566.

(15) Martinez-Ferez, A.; Guadix, A.; Zapata-Montoya, J. E.; Guadix, E. M. Influence of transmembrane pressure on the separation of caprine milk OS from protein by cross-flow ultrafiltration. *Int. J. Dairy Technol.* **2008**, *61*, 333−339.

(16) Mehra, R.; Barile, D.; Marotta, M.; Lebrilla, C. B.; Chu, C.; German, J. B. Novel high-molecular weight fucosylated milk OS identified in airy streams. *PLoS One* **2014**, *5*, e96040.

(17) Urashima, T.; Taufik, E.; Fukuda, K.; Asakuma, S. Recent advances in studies on milk OS of cows and other domestic farm animals. *Biosci., Biotechnol., Biochem.* **2013**, *77*, 455−466.

(18) Meli, F.; Puccio, G.; Cajozzo, C.; Ricottone, G. L.; Pecquet, S.; Sprenger, N.; Steenhout, P. Growth and safety evaluation of infant formulae containing OS derived from bovine milk: a randomized, double blind, noninferiority trial. *BMC Pediatr.* **2014**, *14*, 306−316.

(19) Meyrand, M.; Dallas, D. C.; Caillat, H.; Bouvier, F.; Martin, P.; Barile, D. Comparison of milk OS between goats with and without the genetic ability to synthesize αs1-casein. *Small Ruminant Res.* **2013**, *113*, 411−420.

(20) Martín-Ortiz, A.; Salcedo, J.; Barile, D.; Bunyatratchata, A.; Moreno, F. J.; Martin-García, I.; Clemente, A.; Sanz, M. L.; Ruiz-Matute, A. I. Characterization of goat colostrum OS by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry. *J. Chromatogr. A* **2016**, *1428*, 143−153.

(21) Urashima, T.; Messer, M.; Oftedal, O. T. OS in the Milk of Other Mammals. In *Prebiotics and Probiotics in Human Milk*; McGuire, M., McGuire, M., Bode, L., Eds.; Elsevier: London, UK, 2016; pp 45−139.

(22) Oliveira, D. L.; Wilbey, R. A.; Grandison, A. S.; Duarte, L. C.; Roseiro, L. B. Separation of OS from caprine milk whey, prior to prebiotic evaluation. *Int. Dairy J.* **2012**, *24*, 102−106.

(23) Lara-Villoslada, F.; Debras, E.; Nieto, A.; Concha, A.; Galvez, J.; Lopez-Huertas, E.; Boza, J.; Obled, C.; Xuas, J. OS isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. *Clin. Nutr.* **2006**, *25*, 477−488.

(24) Barnett, A. M.; Roy, N. C.; McNabb, W. C.; Cookson, A. L. Effects of a semi − purified OS − enriched fraction from caprine milk on barrier integrity and mucin production of co-culture models of the small and large intestinal epithelium. *Nutrients* **2016**, *8* (267), 1−17.

(25) Nakamura, T.; Kawase, H.; Kimura, K.; Watanabe, Y.; Ohtani, M.; Arai, I.; Urashima, T. Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J. Dairy Sci.* **2003**, *86*, 1315−1320.

(26) Martinez-Ferez, A.; Zapata, J. E.; Guadixa, A.; Almecija, M. C.; Gomez, M.; Guadix, E. M. Obtention of goat milk permeates enriched in lactose-derived oligosaccharides. *Desalination* **2009**, *245*, 730−736.

(27) Claps, S.; Di Napoli, M. A.; Sepe, L.; Caputo, A. R.; Rufrano, D.; Di Trana, A.; Annicchiarico, G.; Fedele, V. Sialyloligosaccharides content in colostrum and milk of two goat breeds. *Small Ruminant Res.* **2014**, *121*, 116−119.

(28) Fong, B.; Ma, K.; McJarrow, P. Quantification of bovine milk OS using liquid chromatography-selected reaction monitoring-mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 9788−9795.

(29) Liu, Z.; Moate, P.; Cocks, B.; Rochfort, S. A simple LC-MS method for quantification of major free OS in bovine milk. *J. Agric. Food Chem.* **2014**, *62*, 11568−11574.

(30) Brokl, M.; Hernandez-Hernandez, O.; Soria, A. C.; Sanz, M. L. Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. *J. Chromatogr. A* **2011**, *1218*, 7697−7703.

(31) Hernández-Hernández, O.; Calvillo, I.; Lebrón-Aguilar, R.; Moreno, F. J.; Sanz, M. L. Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides. *J. Chromatogr. A* **2012**, *1220*, 57−67.

(32) Martín-Sosa, S.; Martín, M. J.; García-Pardo, L. A.; Hueso, P. Sialyloligosaccharides in human and bovine milk and in infant formulas: Variations with the progression of lactation. *J. Dairy Sci.* **2003**, *86*, 52−59.

(33) Newburg, D. S. Glycobiology of human milk. *Biochemistry* **2013**, *78*, 771−785.

(34) Crisa, A.; Ferre, F.; Chillemi, G.; Moioli, B. RNA-sequencing for profiling goat milk transcript in colostrum and mature milk. *BMC Vet. Res.* **2016**, *12*, 264−284.

(35) Claps, S.; Antonietta Di Napoli, M.; Rocchina Caputo, A.; Rufrano, D.; Sepe, L.; Di Trana, A. Factor affecting the 3′ sialyllactose, 6′ sialyllactose and disialyllactosecontent in caprine colostrum and milk: Breed and parity. *Small Ruminant Res.* **2016**, *134*, 8−13.

(36) Formiga de Sousa, Y. R.; da Silva Vasconcelos, M. A.; Costa, R. G.; Anderson de Azevedo Filho, C.; Prado de Paiva, E.; de Cássia Ramos do Egypto Queiroga, R. Sialic acid content of goat milk during lactation. *Livest. Sci.* **2015**, *177*, 175−180.

(37) Sundekilde, U. K.; Barile, D.; Meyrand, M.; Poulsen, N. A.; Larsen, L. B.; Lebrilla, C. B.; German, J. B.; Bertram, H. C. Natural variability in bovine milk OS from danish Jersey and Holstein-Friesian breeds. *J. Agric. Food Chem.* **2012**, *60*, 6188−6196.

(38) McJarrow, P.; van Amelsfort-Schoonbeek, J. Bovine sialyl OS: seasonal variations in their concentrations in milk, and a comparison of the colostrums of Jersey and Friesian cows. *Int. Dairy J.* **2004**, *14*, 571−579.

(39) Tao, N.; Ochonicky, K. L.; German, J. B.; Donovan, S. M.; Lebrilla, C. B. Structural determination and daily variations of porcine milk oligosaccharides. *J. Agric. Food Chem.* **2010**, *58*, 4653−4659.

(40) Aldredge, D. L.; Geronimo, M. R.; Hua, S.; Nwosu, C. C.; Lebrilla, C. B.; Barile, D. Annotation and structural elucidation of bovine milk OS and determination of novel fucosylated structures. *Glycobiology* **2013**, *23*, 664−676.

DOI: 10.1021/acs.jafc.6b05104
J. Agric. Food Chem. 2017, 65, 3523−3531

(41) Albrecht, S.; Lane, J. A.; Marin, K.; Al Busadah, K. A.; Carrington, S. D.; Hickey, R. M.; Rudd, P. M. A comparative study of free OS in the milk of domestic animals. *Br. J. Nutr.* **2014**, *111*, 1313−1328.
(42) Salcedo, J.; Frese, S. A.; Mills, D. A.; Barile, D. Characterization of porcine milk OS during early lactation and their relation to the fecal microbiome. *J. Dairy Sci.* **2016**, *99*, 7733−7743.

DOI: 10.1021/acs.jafc.6b05104
J. Agric. Food Chem. 2017, 65, 3523−3531

4. COMPORTAMIENTO CROMATOGRÁFICO Y ESPECTROMÉTRICO EN LC-MS PARA CARBOHIDRATOS PREBIÓTICOS

Si deseas hacer un pastel de manzana desde cero, primero tendrás que inventar el universo.

Carl Sagan

4. PREFACIO

En la sección anterior se ha mostrado la utilidad de la cromatografía de líquidos y de gases acoplada a espectrometría de masas para el análisis de los oligosacáridos de la leche de cabra. La leche de cabra, como se ha indicado, es una mezcla muy compleja de oligosacáridos, tanto ácidos como neutros, con distintas unidades monoméricas, grados de polimerización y enlaces glicosídicos. Sin embargo, existen otras mezclas de carbohidratos, aparentemente más sencillas, pero que su análisis entraña también gran dificultad. Son, por ejemplo, mezclas de oligosacáridos prebióticos como GOS, GEOS, etc. o mezclas presentes en plantas, alimentos (como miel, jarabes, etc.) constituidas por oligosacáridos neutros (DP2-DP6) con unidades de glucosa, fructosa y/o galactosa unidas por distintos enlaces glicosídicos. La **cromatografía de líquidos** suele ser la técnica de elección para el análisis de estos oligosacáridos (Wang y col., 2015; Rodríguez-Sánchez y col. 2015). En estos casos se hace necesaria la selección del modo de operación y la optimización de la metodología correspondiente que permita la mejor separación de los compuestos de interés. Hasta el momento son pocos los estudios que permiten la comparación de distintos modos de operación con el fin de seleccionar el más apropiado para cada caso concreto. En este sentido, Brokl y col. (2011) evaluaron el empleo de 4 modos de operación (HILIC, cromatografía con columnas de carbón grafitizado (PGC), RP y HPAEC) para el análisis de trisacáridos, observando que la HILIC y, la cromatografía con PGC eran los modos más prometedores para el análisis de mezclas complejas de carbohidratos neutros.

Por otro lado, y como se ha comentado anteriormente, cuando se trabaja con LC su acoplamiento a la **espectrometría de masas en modo tándem** (**MS^n**) ofrece una mayor información estructural que otros detectores. Sin embargo, en el caso de las mezclas de carbohidratos, en las que se encuentran presentes distintos isómeros, los fragmentos de masas obtenidos por MS^n son prácticamente similares para muchos de ellos, siendo difícil su diferenciación. Por tanto, la búsqueda de iones diagnóstico que permitan la identificación tentativa de dichos compuestos presenta un gran interés. En la **Sección 4.1** se incluye un trabajo titulado “Chemical structure and liquid chromatographic-tandem mass spectrometric data relationship of di- and trisaccharides” de Martín-Ortiz y col. (enviado para publicación a una revista del área de Química Analítica) que tuvo como objetivo la comparación de métodos de LC, basados en el empleo de diferentes modos de operación, para el análisis de di- y trisacáridos y la evaluación de los fragmentos obtenidos mediante MS^n que permitan establecer relaciones con su estructura química.

Sin embargo, a pesar del alto poder de resolución de la LC, la separación de mezclas complejas de carbohidratos no es completa. En este sentido, la cromatografía de líquidos multidimensional (MD-LC) es considerada una interesante alternativa a la 1D LC para el análisis de muestras complejas. La mayor capacidad de pico (n_C) obtenida junto al elevado poder de separación e identificación derivado de la combinación de dos o más procesos de separación independientes, hacen de la MD-LC una técnica muy prometedora para el análisis de alimentos (Cacciola y col., 2017). Sin embargo, hasta el momento no existen antecedentes del uso de esta técnica para el análisis de carbohidratos.

En el caso de la **cromatografía de líquidos bidimensional completa** (**LC×LC**), a la vez que se produce la separación en 1D, se van colectando pequeñas fracciones a la salida de la columna que a su vez se van transfiriendo automáticamente a la segunda dimensión. Estas dos dimensiones deben presentar diferentes mecanismos de retención, a ser posible, independientes, para dar una separación ortogonal. De este modo, compuestos que coeluyen y no pueden ser separados en una primera dimensión se podrán resolver en la segunda (Herrero y col., 2009). Además, si los mecanismos de retención en ambas dimensiones se seleccionan cuidadosamente, la selectividad proporcionada permite separaciones estructuradas en el plano bidimensional, de gran utilidad para la identificación de "desconocidos" y especialmente interesante en el caso de muestras complejas que contienen componentes con un cierto grado de similitud estructural (Cacciola y col., 2017; Pirok y col., 2018).

La **instrumentación** para LC×LC consiste básicamente en tres partes: i) una primera dimensión compuesta por un inyector, una bomba de LC y una columna cromatográfica, que puede ir o no acoplada a un detector; ii) una segunda dimensión con otra bomba de LC, columna y detector; y iii) una interfase o modulador que conecta ambas dimensiones y es la encargada de ir continuamente recogiendo el eluyente de la ^{1}D y transferirlo a la segunda dimensión (Figura 1).

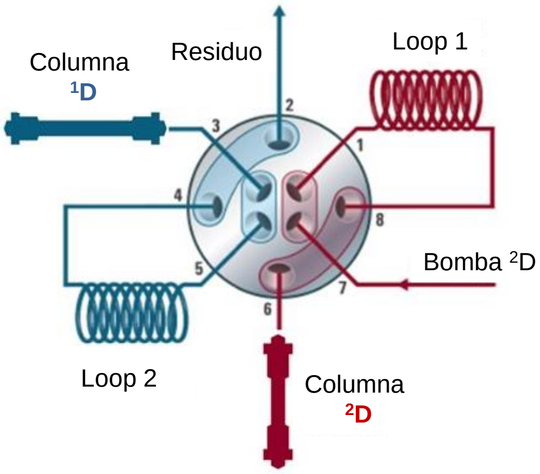

Figura 4.1: Válvula de la interfase en un sistema de LC×LC

Un factor crítico a la hora de desarrollar una metodología por LC×LC es la **selección de columnas y fases móviles** a utilizar, que proporcionen una alta ortogonalidad en la separación. De forma ideal, la separación en ^{1}D debería permitir obtener la mayor separación posible de la mezcla, mientras que ^{2}D debería ser rápida, eficaz y compatible con el detector elegido. Por tanto, las columnas ^{1}D son generalmente largas (100-250 mm), mientras que las columnas para ^{2}D son cortas (50 mm o menos). La elección de diámetros de las columnas está estrechamente ligada al ensanchamiento de la banda en la separación ^{2}D y al factor de dilución (relación entre la concentración de analito en la muestra inyectada y a la entrada del detector). Se ha observado que una relación óptima de diámetros entre las dos columnas ($^{2}d_{c}/^{1}d_{c}$) para conseguir una apropiada separación de los compuestos es entre 4 y 7 (Pirok et al., 2018). También es importante a la hora de desarrollar un método mediante LC×LC, la morfología de la fase estacionaria empleada en cada dimensión. Actualmente, la mayoría de los métodos desarrollados emplean columnas con partículas de sílice totalmente porosas, con distintos grupos funcionales dependiendo del modo de operación elegido, aunque también las columnas monolíticas o con partículas “core-shell” se usan en determinadas aplicaciones (Pirok et al., 2018).

Los modos de operación existentes en LC son muy variados (RPLC, NPLC, HILIC, GCC, SEC, IEC, etc), siendo múltiples las posibles configuraciones en LC×LC. Debido a su versatilidad, la alta capacidad de separación, rapidez de equilibrado y selectividad hacia gran variedad de compuestos, la RP es el modo de operación más empleado en la segunda dimensión (Li y col., 2015). Otras ventajas adicionales de la RPLC son que permite trabajar a altas temperaturas para mejorar la separación y que utiliza fases móviles compatibles con MS, por lo que su uso está muy extendido (Pirok y col., 2018). Sin embargo, como se ha comentado en la Introducción de esta Memoria, la aplicabilidad de este modo de operación para el análisis de carbohidratos es muy limitada debido a la poca retención de los mismos (Brokl y col., 2011), siendo necesario recurrir a reacciones previas de derivatización que mejoren las propiedades cromatográficas.

Las combinaciones NP×RP, RP×RP, HILIC×RP son las más empleados para el análisis de alimentos (Herrero y col., 2009). La mayoría de las aplicaciones han estado centradas en el análisis de triacilgliceroles, fosfolípidos, carotenoides, péptidos y polifenoles (Cacciola y col., 2017). Sin embargo, hasta el momento no se han estudiado posibles aplicaciones de la LC×LC para el análisis de carbohidratos.

La **interfase** también es un punto crítico para conseguir un análisis mediante LC×LC satisfactorio puesto que es donde se produce la interacción física entre las dos dimensiones. En este punto hay que controlar una serie de parámetros como es la relación entre el flujo de la ^{1}D y el volumen de inyección en la ^{2}D, que viene determinado por el tiempo entre modulaciones (Montero, 2017; Pirok y col., 2018).

Las configuraciones de interfases más utilizadas, debido a su gran simplicidad y reproducibilidad, suelen estar basadas en válvulas de varios puertos (8-12) con dos posiciones que se van alternando para recibir el eluyente de la ^{1}D y transferirlo a la ^{2}D (Montero, 2017). En general, estas válvulas van equipadas con dos bucles de carga (loops) de idéntico volumen interno conectados uno a la salida de la columna de la ^{1}D y el otro a la bomba de la 2D y a la entrada de la segunda columna. .

Uno de los principales problemas que pueden surgir a la hora de desarrollar metodologías por LC×LC es que existan incompatibilidades entre las fases móviles de ambas dimensiones. Diferencias en la fuerza del disolvente o distinta viscosidad pueden provocar que la fracción transferida desde la ^{1}D sufra una importante distorsión de pico y se produzcan pérdidas de poder de resolución en la ^{2}D. Además, al estar implicados dos procesos cromatográficos, existen problemas de dilución de la muestra que puede afectar a la sensibilidad en la detección (Stoll y Carr, 2017).

Para intentar minimizar estos problemas, una alternativa es el uso de “columnas de atrapamiento” o “trampas” (trapping columns) sustituyendo los bucles de carga de la interfase, que presenta como ventaja el introducir un mecanismo de enfoque previo a su inyección en la ^{2}D. Así, los analitos son retenidos por la fase estacionaria de la trampa, produciéndose un efecto de concentración, entrando como bandas estrechas en la columna de la segunda dimensión (Cacciona y col., 2007). Normalmente, estas trampas se seleccionan para que coincidan con la selectividad de la columna de ^{2}D pero que no proporcionen una retención ni muy fuerte ni muy débil de los analitos, de forma que la fase móvil de la segunda dimensión desorba los compuestos atrapados de forma eficiente (Egeness y col., 2016). Sin embargo, pueden continuar existiendo problemas de incompatibilidad de las fases que dificulten que la retención de algunos analitos sea de forma eficiente (Montero, 2017).

Una mejora a este sistema, conocida como “modulación activa” (active modulation), consiste en la introducción de una bomba adicional con un disolvente más débil entre la salida de la ^{1}D y la entrada de las columnas de atrapamiento de la interfase (Figura 2). De este modo, se reduce la fuerza del eluyente de la ^{1}D a la vez que se aumenta la retención de los analitos en la columna de atrapamiento, produciéndose un fenómeno de enfoque. Así, los analitos retenidos en la trampa, serán eluídos por la fase móvil inicial de la ^{2}D, completamente compatible con la fase estacionaria de la segunda dimensión, traduciéndose en picos más simétricos, más estrechos y con una mayor eficiencia. Además, este sistema permite utilizar columnas en ^{1}D con un diámetro interno mayor, pudiendo cargar un mayor volumen de muestra lo que implica un aumento de la sensibilidad (Montero, 2017; Pirok y col., 2018).

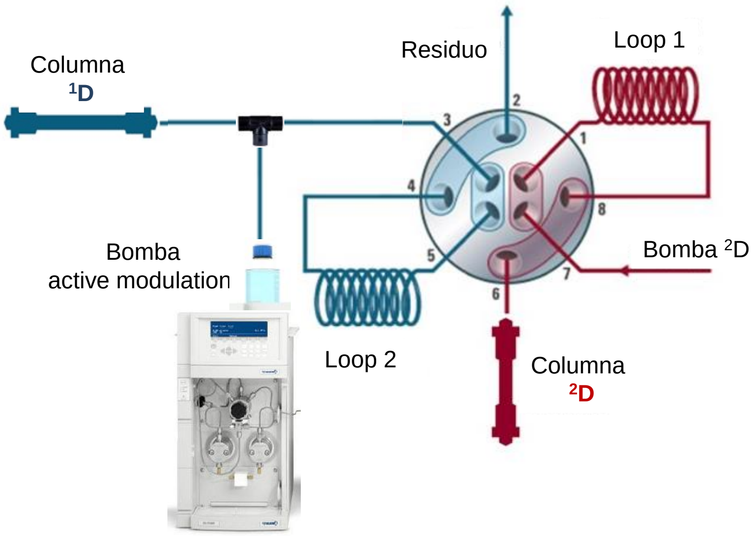

Figura 4.2: Mecanismo de la modulación activa acoplada a LC×LC

Existen otras configuraciones de interfases basadas en la evaporación asistida por vacío o en el uso de moduladores térmicos. Aunque se ha demostrado que son efectivas para resolver ciertos problemas como son los efectos de dilución o de ensanchamiento de la banda de la ^{2}D, presentan ciertas limitaciones. Así, en el caso de los moduladores por evaporación a vacío existe un alto riesgo de pérdida de analitos (Tian y col., 2008) mientras que en el caso de los moduladores térmicos, la principal limitación es que los análisis de ^{2}D están condicionados por los ciclos del modulador, no pudiendo realizar análisis en tiempos menores a 40 s (Creese y col., 2017).

Por tanto, en la **Sección 4.2** se ha desarrollado, por primera vez, un método mediante LC×LC para el análisis de mezclas de di- y trisacáridos que posteriormente ha sido aplicado con éxito para el análisis de OS prebióticos comerciales. Dichos carbohidratos fueron previamente derivatizados empleando 4-aminobenzoato de etilo como reactivo de derivatización, permitiendo así su detección por UV y su separación en RPLC. Estos resultados se han publicado en el artículo "Separation of di- and trisaccharide mixtures by comprehensive two-dimensional liquid chromatography. Application to prebiotic oligosaccharides" de Martín-Ortiz y col. en la revista Analytica Chimica Acta, 1060, (2019), 125-132. Estos estudios pueden considerarse pioneros en este campo y abren una nueva vía de investigación sobre el análisis de carbohidratos.

Bilbiografía:

Bank, S., Kapkova, P., (2012) Liquid chromatography-mass spectrometry of carbohydrates derivatized with biotinamidocaproylhydrazide. Carbohydrates, 55-80.

Brokl, M., Hernández-Hernández, O., Soria, A.C., y Sanz, M.L. (2011) Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. Journal of Chromatography A, 1218, 7697-7703.

Cacciola, F., Jandera, P., Hajdú, Z., Cesla, P., Mondello, L., (2007) Comprehensive two-dimensional liquid chromatography with parallel gradients for separation of phenolic and flavone antioxidants. Journal of Chromatography A, 1149, 73–87.

Creese, M.E., Creese, M.J., Foley, J.P., Cortes, H.J., Hilder, E.F., Shellie, R.A., Breadmore, M.C. (2017) Longitudinal on-column thermal modulation for comprehensive two-dimensional liquid chromatography. Analytical Chemistry, 89, 1123-1130.

Egeness, M.J., Breadmore, M.C., Hilder, E.F., Shellie, R.A., (2016) The modulator in comprehensive two-dimensional liquid chromatography. LC GC Europe, 29 (5), 268-276.

Herrero, M., Ibáñez, E., Cifuentes, A., Bernal, J., (2009) Multidimensional chromatography in food analysis. Journal of Chromatography A, 1216, 7110–7129.

Li, D., Jakob, C., Schmitz, O.J., (2015) Practical considerations in comprehensive two-dimensional liquid chromatography systems (LC×LC) with reversed-phases in both dimensions. Analytical and Bioanalytical Chemistry, 407, 153–167.

Montero, L., (2017) Development of advanced analytical methods for the analysis of complex food matrices by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry (LC × LC-MS). Tesis doctoral. Universidad Autónoma, Madrid, España.

Pirok, B.W.J., Gargano, A.F.G., Schoenmakers, P.J. (2018) Optimizing separations in online comprehensive two-dimensional liquid chromatography, Journal of Separation Science, 41(1), 68-98.

Rodríguez-Sanchez, S., Garcia-Sarrio, M.J., Quintanilla-Lopez, J.E., Soria, A.C., Sanz, M.L., (2015) Analysis of iminosugars and other low molecular weight carbohydrates in Aglaonema sp extracts by hydrophilic interaction liquid chromatography coupled to mass spectrometry. Journal of Chromatography A, 1423, 104-110.

Stoll, D. R., Carr, P. W., (2017) Two-Dimensional Liquid Chromatography: a State of the Art Tutorial. Analytical Chemistry 89, 519–531.

Tian, H., Xu, J., & Guan, Y. (2008). Comprehensive two-dimensional liquid chromatography (NPLC x RPLC) with vacuum-evaporation interface. Journal of Separation Science, 31, 1677–1685.

Wang, S., Guo, Q., Wang, L., Lin, L., Shi, H., Cao, H., Cao, B., (2015) Detection of honey adulteration with starch syrup by high performance liquid chromatography. Food Chemistry, 172, 669-674.

Sección 4.1: Avances en la elucidación de la estructura de carbohidratos de bajo peso molecular mediante LC-MS^n

“Advances in structure elucidation of low molecular weight carbohydrates by liquid chromatography-tandem mass spectrometry analysis”

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Advances in structure elucidation of low molecular weight carbohydrates by liquid chromatography-tandem mass spectrometry analysis

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Abstract

Oligosaccharides are gaining importance because of their beneficial properties in human health. They appear as complex mixtures of different monomeric units, glicosidic linkages and degrees of polymerization (DP) being the most abundant DP2 and DP3. Liquid chromatography-mass spectrometry is one of the most used techniques for their analysis, however, there are scarce information correlating their chemical structure, mass spectra and chromatographic data. In this work, chromatographic parameters for 17 disaccharides were obtained using PGC and HILIC columns. Moreover, MS^2 fragmentation of these disaccharides with different linkages and monomeric units (glucose, galactose, mannose and fructose) was studied. The correlation of chromatographic retention data and characteristic *m/z* ions observed by MS^n with statistical analysis was successful in establishing some specific criteria that allowed the characterization of trisaccharides with different structural features.

Keywords: disaccharides, trisaccharides, liquid chromatography-mass spectrometry, glycosidic linkage, monomeric units

1. Introduction

Oligosaccharides are usually found as complex mixtures of carbohydrates with several monosaccharide units (mainly galactose, glucose and/or fructose) and degrees of polymerization (DP), usually from DP2 to DP7, linked through different glycosidic linkages. In general, those carbohydrates of DP2 (disaccharides) and DP3 (trisaccharides) are the most abundant in both synthetic mixtures and nature.

Several beneficial properties for consumers are attributed to different oligosaccharides, mainly associated to their prebiotic effect and their antiadhesive activity [1,2]; then, a detailed characterization of their chemical structures is demanding. However, structural characterization of di- and trisaccharides in natural samples is not a straightforward task considering the low availability of commercial oligosaccharide standards, the similarity of their structure, their presence in complex mixtures and their different abundances.

Gas chromatography coupled to mass spectrometry (GC-MS) is one of the most commonly used techniques for the characterization of di- and trisaccharides [3,4,5], considering its resolution power and identification capacity provided by the electron impact (EI) ionization. However, a derivatization step, previous to oligosaccharide analysis, is mandatory.

High performance liquid chromatography (LC) coupled to mass spectrometry (MS) is also an appropriated technique for the analysis of carbohydrates. Among the different LC operation modes, porous graphitized carbon (PGC) liquid chromatography and hydrophilic interaction liquid chromatography (HILIC) have emerged as efficient alternatives for the separation of polar compounds [6,7,8,9]. In particular, PGC-LC seems promising for the separation of carbohydrates with the same molecular weight and it has been proposed for the separation of isomeric structures [10,11]. HILIC provides satisfactory separations among oligosaccharides with different degree of polymerization and monomeric units [12,13,14,15]. Both operation modes are easily coupled to mass spectrometry and enable the analysis of carbohydrates without derivatization [15]. The soft ionization used in LC-MS joined to quadrupole analyzers limits the utility of this technique considering the scarce structural information provided (i.e. only the quasimolecular ion is obtained). Then, the use of tandem MS analyzers is required to determine glycosidic linkage positions and monosaccharide sequences [13,16,17,18,19]. Electrospray ionization (ESI) can operate in positive and negative ion modes. Negative ESI mode induces a good cross-ring fragmentation and provides reliable structural information for high molecular weight carbohydrates, however, at the expense of achieving a low sensitivity [15]. On the contrary, positive ESI mode can be a good alternative for the analysis of low molecular weight carbohydrates such as di- and trisaccharides [13,19,20]. Hernández-Hérnandez et al. [13] use HILIC-MS^n at positive ESI mode for the tentative identification of complex galactooligosaccharides mixtures (up to DP4). However, to the best of our knowledge scarce information on correlation among chemical structures, mass spectra and LC data of di- and trisaccharides with different monomeric units and glycosidic linkages can be found in the literature. Then, in this work, reliable information about chromatographic data (using PGC and HILIC columns) and characteristic MS^2 fragmentation of several disaccharides with different linkages and monomeric units (glucose, galactose and fructose) was obtained to establish some specific criteria that allowed the characterization of higher molecular weight carbohydrates. This information was useful for the study of trisaccharides with different structures.

2. Materials and methods

2.1. Standards

1,3-Galactobiose (α-Gal-[1→3]-Gal), 1,4-galactobiose (β-Gal-[1→4]-Gal), 1,6-galactobiose (β-Gal-[1→6]-Gal) were acquired from Dextra Laboratories (Reading, UK), whereas trehalose (α-Glc-[1→1]-Glc), kojibiose (α-Glc-[1→2]-Glc), kojitriose (α-Glc-[1→2]$_2$-Glc), nigerose (α-Glc-[1→3]-Glc), lactose (β-Gal-[1→4]-Glc), laminaribiose (β-Glc-[1→3]-Glc), laminaritriose (β-Glc-[1→3]$_2$-Glc), isomaltose (α-Glc-[1→6]-Glc), maltose (α-Glc-[1→4]-Glc), maltotriose (α-Glc-[1→4])$_2$-Glc), cellobiose (β-Glc-[1→4]-Glc), lactulose (β-Gal-[1→4]-Fru), trehalulose (α-Glc-[1→1]-Fru), sucrose (α-Glc-[1→2]-Fru), turanose (α-Glc-[1→3]-Fru), maltulose (α-Glc-[1→4]-Fru), leucrose (α-Glc-[1→5]-Fru), palatinose (α-Glc-[1→6]-Fru), planteose (α-Gal-[1→6]-Fru-α-[2→1]-Glc), theanderose (α-Glc-[1→6]-Glc-α-[1→2]-Fru), lactosucrose (β-Gal-[1→4]-Glc-α-[1→2]-Fru), gentianose (β-Glc-[1→6]-Glc-α-[1→2]-Fru), galactotriose (β-Gal-[1→3]-Gal-β-[1→4]-Gal), 1,2-manobiose (α-Gal[1→2]-β-Man), 1,6-manobiose (α-Gal[1→6]-β-Man), melibiose (α-Gal-(1→6)-Glc) and gentibiose (β-Glc-[1→6]-Glc) were obtained from Sigma (St. Louis, USA).

2.2. HPLC–MS

Analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (5 µL) were injected using a Rheodyne 7725 valve. Two different columns were evaluated:

(i) an ethylene bridge hybrid (BEH) column with trifunctionally bonded amide phase (XBridge column; 150 × 4.6 mm; 3.5 µm particle size, 135 A° pore size, Waters, Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1}. The separation of di- and trisaccharides was optimized by using different binary gradients of acetonitrile as solvent A and water as solvent B (both with 0.1% NH_4OH) using a hydrophilic interaction liquid chromatography (HILIC) mode. Final conditions were 0 min, 80% A and 31 min, 50% A.

(ii) a porous graphitic carbon (PGC) column (Hypercarb® 100 mm × 2.1 mm; 5 µm; Thermo Fisher Scientific, Barcelona, Spain). After binary gradient optimization, deionized water as solvent A and ACN as solvent B, both with addition of 0.1% NH_4OH, and the following gradient at a flow rate of 0.4 mLmin^{-1}: 0 min, 0% B; 1 min, 0% B; 20 min, 20 % B; 21 min, 90% and kept for 7 min.

The temperature of elution was kept at 35 °C in all cases. ESI source was operated under positive polarity using the following MS parameters: nebulizing gas (N_2) pressure 276 kPa, nitrogen drying gas at a flow rate of 12 L min^{-1} and 300 °C and capillary voltage of 4000 V. Ions corresponding to mono-sodiated adducts $[M+Na]^+$ of the oligosaccharides under analysis were monitored in SIM mode using default variable fragmentor voltages: 365.0 and 527.0 *m/z* values for di- and trisaccharides, respectively. Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Different chromatographic parameters were considered for di- and trisaccharides eluting in each column such as retention times (t_R), peak width at middle height (w_h), peak symmetry, calculated as the ratio of the front to back widths (at 50% of the peak height), resolution between two consecutive peaks (Rs, calculated as $2(t_{R2} - t_{R1})/(w_{b1} + w_{b2})$, where 1 and 2 refer to two consecutive eluting carbohydrates and w_b is the peak width at base), height equivalent to a theoretical plate (HETP) and peak capacity (n_c) defined as the maximum number of non-overlapping peaks in a given interval, was calculated as follows: $n_c = 1 + (t_g/ w_b)$, where t_g is the difference in t_R within a given interval and w_b is the mean peak width value at base.

2.3. MS^n

A Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface was used. Sample injections (20 µL) were carried out by a Finnigan Surveyor autosampler. All instruments (Thermo Fisher Scientific, San José, CA, USA), and data acquisition were managed by Xcalibur software (1.2 version; Thermo Fisher Scientific).

The mass spectrometer spray voltage was set at 4.5 kV and the heated capillary temperature at 290ºC. Nitrogen (99.5% purity) was used as sheath (0.9 L min^{-1}) and auxiliary (9 L min−1) gas, and helium (99.9990% purity) as the collision gas in the collision induced dissociation (CID) experiments. Mass spectra were acquired in the positive ion mode. Fragmentation behavior of the oligosaccharides was studied by infusing a solution of each oligosaccharide (10 µg mL^{-1} in MeCN:water, 60:40, v/v) at a flow rate of 10 µL min^{-1} using the syringe pump included in the instrument and mixing it with 100 µL min^{-1} of MeCN:water (60:40, v/v) both with 0.1% ammonium hydroxide by means of a zero-dead volume T-piece. Sheath and auxiliary gases were set at 0.6 and 6 L min^{-1}, respectively. CID experiments were carried out by isolating each $[M+Na]^+$ ion in the ion trap (isolation width 1.0 *m/z*), and subjecting them to a normalized collision energy (NCE%) selected to preserve a signal of the precursor ion in the order of 5%. The process was repeated up to two times by successive isolation (isolation width 1.0 *m/z*) of the generated ions corresponding to the loss of a monosaccharide unit (loss of 162 u).

2.4. Statistical analysis

Stepwise discriminant analysis was carried out using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA). The F to enter criteria was set as 4.

3. Results and discussion

3.1. Chromatographic data of disaccharides

Different chromatographic gradients of acetonitrile and water both with 0.1% NH_4OH, adapted from Brokl et al. [12], were assayed for BEH-amide and PGC columns. Optimal conditions, as indicated in Materials and Methods section, were selected as a trade-off among shortest t_R, best peak symmetry, higher Rs, and smallest w_h. As previously observed, the use of NH_4OH avoids the appearance of split peaks corresponding to α and β isomers [13].

Tables 1 and 2 show the chromatographic parameters obtained for 17 disaccharides with different glycosidic linkages and monomeric composition (glucose, galactose and fructose), eluting in both PGC and BEH-amide columns. Under these chromatographic conditions di- and trisaccharides eluted between 6.4 and 10.6 min in PGC column and between 20.4 and 25.4 min in BEH-amide column. The great retention of carbohydrates on the BEH-amide column has been explained in previous works due to the contribution of strong hydrogen bonding effects between the amide group of the stationary phase and hydroxyl groups of carbohydrates [13]. As expected, different elution orders of the investigated carbohydrates were observed in function of their structural features. Thus, glucosyl-fructoses eluted before glucosyl-glucoses and these before galactosyl-galactoses in the BEH-amide column. On the contrary, no specific relationship was observed between monomeric composition and retention time for PGC column.

Regarding glycosidic linkages, disaccharides with linkages 1→6 eluted earlier than those with 1→4 linkages and the same monomeric structure in the PGC column. However, in the BEH-amide column, isomaltose and 6'galactobiose (both aldoses with linkages 1→6) were the most retained disaccharides, even the last one eluted within the trisaccharide area.

In general, acceptable peak width values were obtained in the BEH-amide column for all disaccharides (w_h < 0.61 min), except for galactobioses (w_h: 0.72-1.38 min) and nigerose (w_h: 0.88 min) (Table 2); thinner peaks were in general obtained with the PGC column (w_h < 0.55 min, except for 3'galactobiose and lactose) (Table 1). Good peak symmetry values were also observed with both columns (mean symmetry values: 1.00 in BEH-amide and 1.11 in PGC). On the contrary, peak capacity values were very poor for both columns (approx. 6 and 7 for BEH-amide column and PGC column, respectively). Chromatographic information can be enhanced by the combination of data obtained by both LC operation modes.

3.2. Mass spectrometric data of disaccharides

MS^2 analyses of disaccharides using the ion at *m/z* 365 (sodium adduct) revealed characteristic fragments from the consecutive loss of H_2O and CHOH units for the different glycosidic linkages. In general, fragmentation behavior for glucosyl-glucoses, galactosyl-galactoses and mannosyl-mannoses was similar; however, disaccharides containing a fructose unit gave different *m/z* fragments and were independently considered (Figure 1).

Only slight differences in the relative abundances of characteristic *m/z* fragments could be observed for the aldosyl-aldoses (glucose, galactose and/or mannose containing disaccharides). Mass spectrum of the disaccharide with 1→1 linkage (α,β-trehalose) only showed an abundant fragment at *m/z* 203, corresponding to the loss of a monosaccharide unit, whereas the disaccharides with 1→2 linkage (kojibiose and 1,2-mannobiose) were characterized by the ion at *m/z* 245, corresponding to the loss of 4 CHOH units. Mass spectrum of disaccharides with 1→3 linkage (nigerose and 1,3-galactobiose) was characterized by the high abundance of *m/z* fragment at 347 (corresponding to the loss of a molecule of water) followed by the loss of the monosaccharide unit (*m/z* 203) and the loss of 3 CHOH units (*m/z* 275). In general, *m/z* ion at 305 (corresponding to the loss of 2 CHOH units) was the base peak for disaccharides with 1→4 (maltose, cellobiose, lactose and 1,4-galactobiose) and 1→6 (isomaltose, 1,6-galactobiose and 1,6 mannobiose) linkages. Distinction between these carbohydrates could be done considering the presence of relatively high abundant ions at *m/z* 275 in 1→6 disaccharides (except for 1,6-mannobiose) and a small contribution of *m/z* 245 (losses of 3 and 4 CHOH units, respectively). These results agree with those shown by Zhang et al. [20] and Hernández-Hernández et al. [13] for some of these disaccharides, only differences regarding the abundance of the fragment ions were observed in specific cases (e.g. 3'-galactobiose analyzed by Zhang et al. [20] showed a higher intensity of *m/z* 203). These differences could be due to the mass spectrometer used.

Regarding disaccharides containing a fructose unit, fragment at *m/z* 317 showed to be characteristic of 1-4 linkages and fragment at *m/z* 275 was found to be the base peak for 1→1, 1→3 and 1→6 linkages, whereas ion at *m/z* 203 was characteristic of 1→2 and 1→5 linkages and *m/z* 347 of 1→4 linkages. It is worth to note that palatinose (1,6-Glc-Fru) showed the same fragmentation profile that the aldosyl-aldoses 1→6, although relative abundances of the corresponding ions were different. A similar behavior was observed for maltulose (1,4-Glc-Fru) and lactulose (1,4-Gal-Fru) compared to lactose and 4β-galactobiose. Moreover, sucrose also showed the same fragmentation pattern than the non-reducing aldosyl-aldose (trehalose). To the best of our knowledge, MS data of glucosyl-fructoses have not been previously reported in the literature.

Stepwise discriminant analyses were applied to the 24 standard samples × 7 variables fragment data matrix. Samples were grouped according to the linkage in which the monosaccharide units are attached (1→1, 1→2, 1→3, 1→4, 1→6), to the reducing character and to the presence of monosaccharides moieties (glucose, galactose, fructose, mannose). F-statistic was used to estimate the significance of the relation between the different fragments and the chemical structure. The percentage of samples that were correctly classified was considered a practical criterion of the possibility to recognize a specific feature of the disaccharides using the selected MS fragments. Several simulations to estimate the percentage of correct classification by using randomly selected groups were also carried out, this value was 45% or lower.

Table 3 showed the percentage of correctly classified cases for each structural feature of disaccharides. Only one variable (*m/z* fragment 245; F value 1733, p-level 0.0000) was enough to correctly classified all cases when linkages 1→2 was selected, however, 3 variables (*m/z* 317, 305 and 347; F values from 8.9 to 9.9) were required to classified the 100% of the cases for 1→4 linkages. A high percentage of correct classification (96%) was also achieved when linkages 1→3 were selected as structural feature, requiring 4 variables (*m/z* 347, 317, 305 and 203; F values from 8.8 to 10.9). On the contrary, only 75% of the cases were correctly classified (*m/z* 305 and 275, F values 11.3 and 9.4) when linkages 1→6 were selected as structural feature. Regarding reducing and non-reducing character, 92% of the cases were correctly classified using only *m/z* 203 (F value 25.9); maltose and leucrose could not be correctly classified with this variable, probably due to the high abundance of this *m/z* fragment in MS of these carbohydrates. Regarding monomeric units, high percentage of correct classification was obtained for both fructose and mannose units (87 and 92%, respectively). Only fragment *m/z* 245 was used to classified mannose containing disaccharides, however, its significance was low (F value 7.5 with p-level 0.012), since only two compounds possess this structural feature. Three variables (*m/z* 275, 317 and 305; F values from 6.4 to 11.3) were selected to classify disaccharides constituted by fructose units. Only 75% of glucosyl-glucoses were correctly classified using *m/z* fragment at 335 (F value of 6.4, p-level 0.019), while classification of galactose containing disaccharides was not possible using discriminant analysis.

3.3. Characterization of trisaccharides

Regarding chromatographic data (Table 4), trisaccharides eluted after disaccharides in both columns, with the only exception of 6'galactobiose on the BEH-amide column and cellobiose on the PGC. However, retention order was different in each column. Laminaritriose (glucotriose with 1→3 linkages) was the first to elute in the BEH-amide column and the last in the PGC. Theanderose and gentianose, which only differ in the α and β orientation of the 1→6 linkage, coeluted on the HILIC column (i.e. R_s 0.03) but were clearly separated when the PGC column was used (R_s 8.0). Galactotriose was highly retained on PGC column, and eluted before laminaritriose (glucotriose with 1→3 linkages). As previously indicated, a similar behavior was observed for galactosyl containing disaccharides.

Good peak width values were obtained, in general, for trisaccharides in both columns, with the exception of laminaritriose (w_h 0.75 min) in BEH-amide column. On the contrary, peak symmetry values were worse than those obtained for disaccharides (mean symmetry values of 1.28 for BEH-amide column and 0.86 for PGC column). While peak capacity was low for trisaccharides eluting on BEH-amide column (n_c: 4), higher n_c values were obtained in the PGC column (n_c: 18).

To determine the relationships between MS data and chemical structure, MS^2 and MS^3 fragmentation were obtained for each trisaccharide (Figure 2) using *m/z* 527 (sodium adduct of trisaccharides) and *m/z* 365 as precursor ions, respectively. The previously obtained discriminant function for each structural feature was applied to this data. In general, MS data was useful to classify trisaccharide structures and characteristic *m/z* fragments agreed with those proposed for the different linkages of disaccharides. Only some discrepancies were observed in MS^2 for lactosucrose, theanderose and gentianose. These carbohydrates were correctly classified as non-reducing sugars (high abundance of *m/z* ion at 365 corresponding to the neutral loss of $C_6H_{10}O_5$), but considering the similarities in the MS fragmentation of non-reducing glucosyl-glucoses (trehaloses) and glucosyl-fructoses (sucrose), the structure of the non-reducing moiety of these trisaccharides could not be confirmed. However, the MS^3 data was useful to unequivocally characterize these carbohydrates (1→4 linkage for lactosucrose and 1→6 linkages for theanderose and gentianose). Another discrepancy was observed for kojitriose; both MS^2 and MS^3 were dominated by fragments corresponding to the neutral loss of 4 CHOH units (ions at *m/z* 407 and 245, respectively), characteristic of the kojibiose, but also showed the contribution of the neutral loss of $C_6H_{10}O_5$ units (ions at *m/z* 365 and 203, respectively) typical of 1,2-mannobiose. Then this carbohydrate could erroneously be classified a mannotriose with 1→2 linkages. Other trisaccharides were correctly characterized by MS^2 and MS^3 data as it is shown in Figure 2.

4. Conclusions

In this work, new criteria based on the combination of retention data on HILIC and PGC columns and characteristic *m/z* fragment ions observed by MS^n have been established for the rapid tentative structural characterization of trisaccharides. This information could be useful in future studies for the characterization of carbohydrates with different polymerization degrees present in complex mixtures.

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References

[1] Oliveira, D.L., Wilbey, R.A., Grandison, A.S., Duarte, L.C., Roseiro, L.B., (2012), Separation of oligosaccharides from caprine milk whey, prior to prebiotic evaluation. International Dairy Journal, 24, 102−106.

[2] Newburg, D.S.; Ruiz-Palacios, G.M.; Morrow, A.L., (2005), Human milk glycans protect infants against enteric pathogens, Annual Review of Nutrition, 25, 37-58.

[3] Sanz, M.L., Sanz, J., Martínez-Castro, I., (2002) Characterization of O-trimethylsilyl oximes of disaccharides by gas chromatography-mass spectrometry. Chromatographia, 56, 617.

[4] Brokl, M., Soria, A.C., Martínez-Castro, I., Sanz, M.L., Ruiz-Matute, A.I., (2009) Characterization of O-trimethylsilyl oximes of trisaccharides by gas chromatography–mass spectrometry. Journal of Chromatography A, 1216 (46) 89-4692.

[5] Ruiz-Matute, A. I., Hernandez-Hernandez, O., Rodriguez-Sanchez, S., Sanz, M. L., Martinez-Castro, I., (2011) Derivatization of carbohydrates for GC and GC-MS

analyses. Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences, 879, 17-18, 1226-1240

[6] Robinson, S, Bergström, E., Seymour, M., Thomas-Oates, J., (2007) Screening of Underivatized Oligosaccharides Extracted from the Stems of Triticum aestivum Using Porous Graphitized Carbon Liquid Chromatography−Mass Spectrometry. Analitical Chemistry, 79 (6), 2437–2445.

[7] Moreno, F.J., Carrero-Carralero, C., Hernández-Hernández, O., Sanz, M.L. (2014) Fractionation of food bioactive oligosaccharides. Food oligosaccharides: production, analysis and bioactivity. (Ed) Moreno y Sanz. Wiley Blackwell

[8] Lane, J.A., Hickey, R.M., (2014) Analysis of Bioactive Food-Sourced Oligosaccharides by High-Performance Liquid Chromatography In: Food oligosaccharides: production, analysis and bioactivity. Moreno, FJ; Sanz, ML (Eds.) IFT Press Series, 399-420.

[9] Yan, J., Ding, J., Jin, G., Yu, D., Yu, L., Lone, Z., Guo, Z., Chai, W., Liang, X., (2018) Profiling of Sialylated Oligosaccharides in Mammalian Milk Using Online Solid Phase Extraction-Hydrophilic Interaction Chromatography Coupled with Negative-Ion Electrospray Mass Spectrometry. Analytical Chemistry, 90 (5), 3174-3182.

[10] Lie, A., Pedersen, L.H., (2018) Resolution and signal-to-noise in analysis of carbohydrate isomers by graphitised carbon chromatography with charged aerosol detection. Journal of Chromatography A, 1567, 147-154.

[11] Tonon, K.M., Miranda, A., Abrão, A.C.F.V., Mauro B. de Morais, M.B., Morais, T.B., (2019) Validation and application of a method for the simultaneous absolute quantification of 16 neutral and acidic human milk oligosaccharides by graphitized

carbon liquid chromatography – electrospray ionization – mass spectrometry. Food Chemistry, 274, 691–697.

[12] Brokl, M., Hernández-Hernández, O., Soria, A.C., y Sanz, M.L., (2011) Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. Journal of Chromatography A., 1218, 7697-7703.

[13] Hernandez-Hernandez, O., Calvillo, I., Lebron-Aguilar, R., Moreno, F. J., Sanz, M. L., (2012) Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides. Journal of Chromatography A, 1220, 57-67.

[14] Martin-Ortiz, A., Salcedo, J., Barile, D., Bunyatratchata, A., Moreno, F.J., Martin-Garcia, I., Clemente, A., Sanz, M.L., Ruiz-Matute, A. I., (2016) Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry. Journal of Chromatography A, 1428, 143-153.

[15] Juvonen, M., Kotiranta, M., Jokela, J., Tuomainen, P., Tenkanen, M, (2019) Identification and structural analysis of cereal arabinoxylan-derived oligosaccharides by negative ionization HILIC-MS/MS. Food Chemistry, 275, 176-185.

[16] Asam, MR., Glish, GL, (1997) Tandem mass spectrometry of alkali cationized polysaccharides in a quadrupole ion trap. Journal of the American society for mass spectrometry, 8 (9) 987-995.

[17] Hofmeister, G.E., Zhou, Z., Leary, J.A. (1991) Linkage position determination in lithium-cationized disaccharides – tandem mass-spectrometry and semiempirical calculations. Journal of the American Chemical Society, 113 (16) 5964-5970.

[18] Mischnick, P. (2011) Principio del formulario Learning chemistry-the Agnes-Pockels-Student-Laboratory at the Technical University of Braunschweig, Germany. Analytical and Bioanalytical Chemistry, 400 (6) 1533-1535.

[19] Neri, D.F.M, Balcão, V.M., Cardoso, S.M., Silva, A.M.S., Domingues, M.R.M., Torres, D.P.M., Rodrigues, L.R.M., Carvalho, L.B., Teixeira, J.A.C., (2011) Characterization of galactooligosaccharides produced by b-galactosidase immobilized onto magnetized Dacron. International Dairy Journal, 21, 172-178.

[20] Zhang, Y., Go, E.P., Desaire, H., (2008) Maximizing coverage of glycosylation heterogeneity in MALDI-MS analysis of glycoproteins with up to 27 glycosylation sites. Analytical Chemistry, 80 (9) 3144-3158.

377 **Figure 1**. Relative abundances of characteristic m/z ratios of neutral losses from MS2,
378 selecting m/z 365 as precursor ion, of disaccharides. *Glycosidic linkages, **MS2
379 fragments

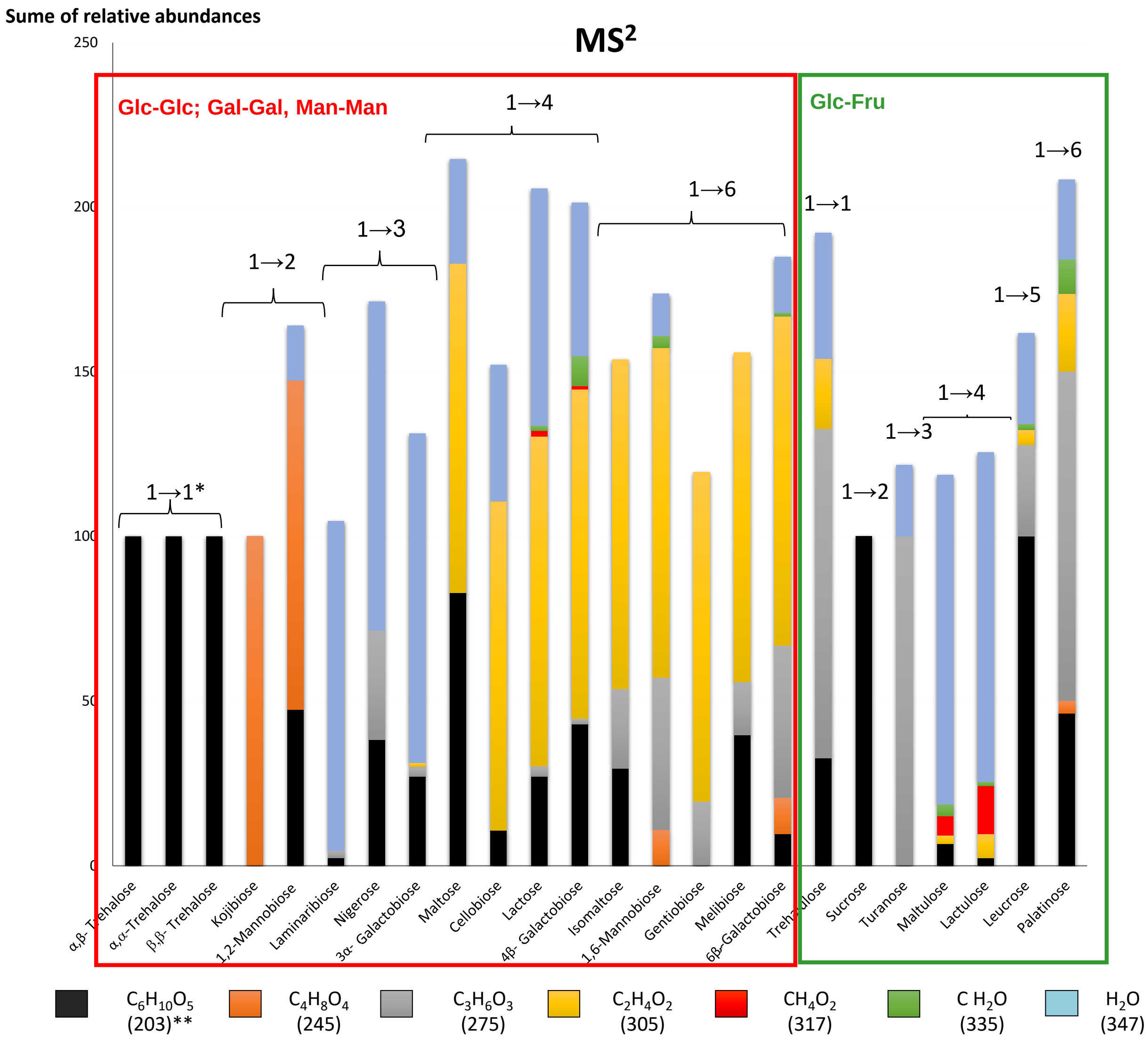

Figure 2. Relative abundances of characteristic m/z ratios of neutral losses from MS2 and MS3 of trisaccharides, selecting m/z 527 and 365 as precursor ions, respectively.

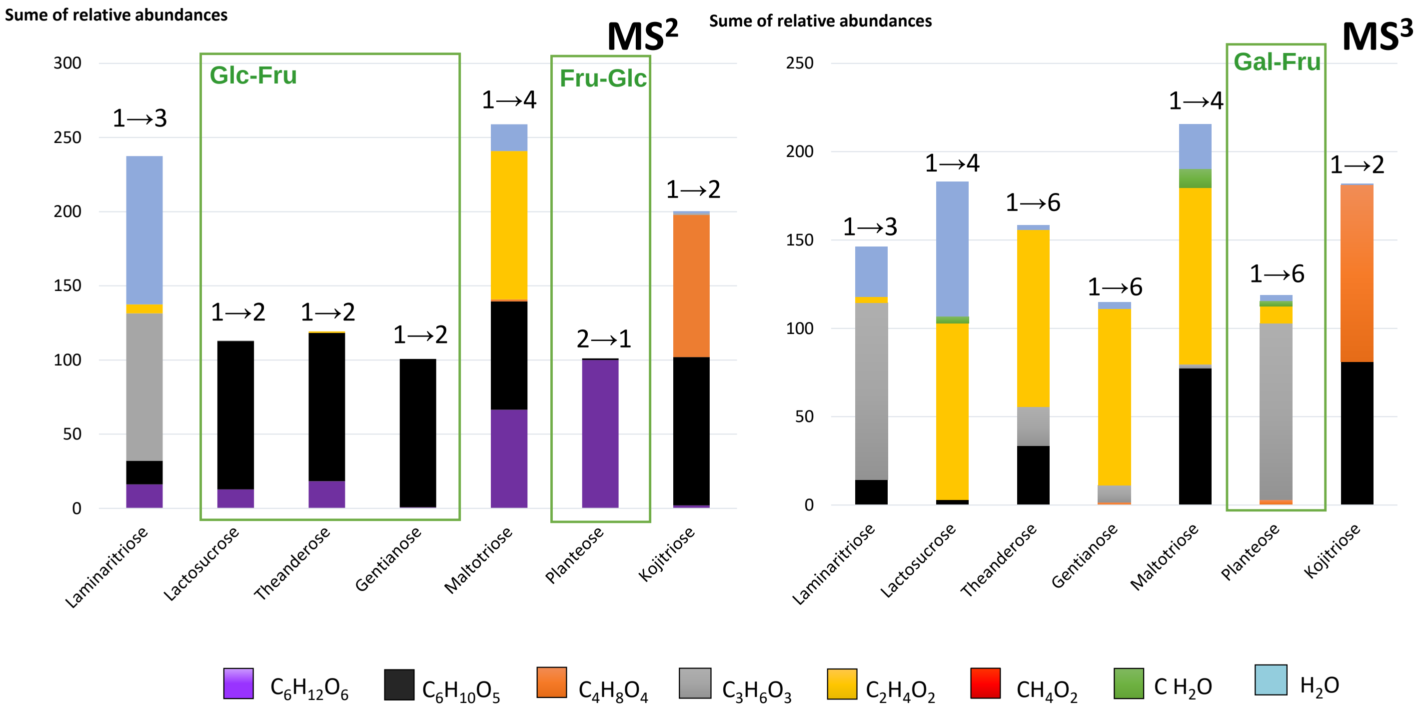

Table 1. Chromatographic parameters for disaccharides eluted on PGC column.

	Monomeric composition	Linkage	Time (min)	w_h (min)	Symmetry	R_s	HETP
Trehalose	Glc-Glc	1→1	6.42	0.40	1.07	0.22	0.034
Turanose	Glc-Fru	1→3	6.58	0.40	1.23	0.64	0.034
Leucrose	Glc-Fru	1→5	6.95	0.28	0.98	0.00	0.015
Palatinosa	Glc-Fru	1→6	6.95	0.33	1.09	0.09	0.020
Lactulose	Gal-Fru	1→4	7.00	0.31	1.39	0.05	0.018
Maltulose	Glc-Fru	1→4	7.02	0.30	1.01	0.47	0.017
Kojibiose	Glc-Glc	1→2	7.36	0.55	0.85	0.24	0.050
Isomaltose	Glc-Glc	1→6	7.54	0.34	1.03	0.25	0.018
6’Galactobiose	Gal-Gal	1→6	7.72	0.51	0.97	0.12	0.039
Trehalulose	Glc-Fru	1→1	7.80	0.31	1.10	0.92	0.015
Maltosa	Glc-Glc	1→4	8.30	0.33	1.05	0.65	0.014
4’Galactobiose	Gal-Gal	1→4	8.73	0.46	1.24	0.09	0.025
Sucrose	Glc-Fru	1→2	8.79	0.30	1.20	0.02	0.010
3’Galactobiose	Gal-Gal	1→3	8.81	1.10	1.57	0.07	0.140
Nigerose	Glc-Glc	1→3	8.89	0.29	1.00	0.16	0.009
Lactose	Gal-Glc	1→4	9.04	0.78	1.05	1.86	0.067
Cellobiose	Glc-Glc	1→4	10.58	0.20	1.08		0.003

Table 2. Chromatographic parameters for disaccharides eluted on BEH-amide column.

	Monomeric composition	Linkage	Time (min)	w_h (min)	Symmetry	R_s	HETP
Sucrose	Glc-Fru	1→2	20.36	0.61	1.30	0.29	0.025
Turanose	Glc-Fru	1→3	20.62	0.46	0.94	0.17	0.013
Palatinose	Glc-Fru	1→6	20.75	0.45	0.76	0.77	0.012
Maltulose	Glc-Fru	1→4	21.33	0.44	0.86	0.32	0.012
Leucrose	Glc-Fru	1→5	21.57	0.42	0.85	0.13	0.010
Maltose	Glc-Glc	1→4	21.68	0.53	1.07	0.06	0.016
Lactulose	Gal-Fru	1→4	21.72	0.40	0.86	0.02	0.009
Cellobiose	Glc-Glc	1→4	21.74	0.51	0.92	0.19	0.015
Nigerose	Glc-Glc	1→3	21.96	0.88	1.33	0.21	0.044
Trehalulose	Glc-Fru	1→1	22.22	0.54	0.99	0.12	0.016
Kojibiose	Glc-Glc	1→2	22.33	0.52	0.87	0.16	0.015
Trehalose	Glc-Glc	1→1	22.45	0.34	0.90	0.16	0.006
4'Galactobiose	Gal-Gal	1→4	22.59	0.72	1.73	0.00	0.028
Lactose	Gal-Glc	1→4	22.59	0.52	0.97	0.11	0.014
3'Galactobiose	Gal-Gal	1→3	22.77	1.38	0.78	0.29	0.099
Isomaltose	Glc-Glc	1→6	23.23	0.52	0.90	1.74	0.014
6'Galactobiose	Gal-Gal	1→6	25.44	0.95	1.03		0.038

Table 3. Percentage of correctly classified cases using discriminant analysis applied to MS fragments of disaccharides.

Structural feature	Cases correctly classified (%)
Glucose unit	75
Galactose unit	25
Fructose unit	87
Mannose unit	92
Reducing/non-reducing character	92
1→2	100
1→3	96
1→4	100
1→6	75

Table 4. Chromatographic parameters for trisaccharides eluted on a PGC column and on a HILIC column.

	Monomeric composition	Linkage	Time (min)	w_h (min)	Symmetry	R_s	HETP
PGC column							
Planteose	Gal-Fru-Glc	1→6; 2→1	10.28	0.16	1.01	1.46	0.002
Maltotriose	Glc-Glc-Glc	1→4; 1→4	11.06	0.47	0.74	1.37	0.016
Theanderose	Glc-Glc-Fru	1→6; 1→2	11.77	0.14	0.90	0.77	0.001
Lactosucrose	Gal-Glc-Fru	1→4; 1→2	11.95	0.14	0.91	0.10	0.001
Kojitriose	Glc-Glc-Glc	1→2; 1→2	12.01	0.48	0.70	3.52	0.015
Gentianose	Glc-Glc-Fru	1→6; 1→2	14.01	0.19	0.91	5.80	0.002
Galactotriose	Gal-Gal-Gal	1→3; 1→4	17.11	0.44	1.02	1.82	0.012
Laminaritriose	Glc-Glc-Glc	1→3; 1→3	18.61	0.53	0.88		0.007
BEH-amide column							
Laminaritriose	Glc-Glc-Glc	1→3; 1→3	23.85	0.75	0.97	0.59	0.027
Lactosucrose	Gal-Glc-Fru	1→4; 1→2	25.11	0.35	1.20	0.44	0.005
Theanderose	Glc-Glc-Fru	1→6; 1→2	25.38	0.39	1.21	0.03	0.006
Gentianose	Glc-Glc-Fru	1→6; 1→2	25.40	0.47	1.21	0.71	0.009
Maltotriose	Glc-Glc-Glc	1→4; 1→4	26.05	0.53	1.01	0.82	0.011
Planteose	Gal-Fru-Glc	1→6; 2→1	26.78	0.53	1.58	0.31	0.011
Kojitriose	Glc-Glc-Glc	1→2; 1→2	27.07	0.57	1.80	0.05	0.012
Galactotriose	Gal-Gal-Gal	1→3; 1→4	27.11	0.44	1.42		0.013

Sección 4.2: Separación de mezclas de di- y trisacáridos mediante LC×LC y su aplicación a carbohidratos prebióticos

“Separation of di- and trisaccharide mixtures by comprehensive two-dimensional liquid chromatography. Application to prebiotic oligosaccharides”

A. Martín-Ortiz, A.I. Ruiz-Matute, M.L. Sanz, F.J. Moreno, M. Herrero

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Separation of di- and trisaccharide mixtures by comprehensive two-dimensional liquid chromatography. Application to prebiotic oligosaccharides

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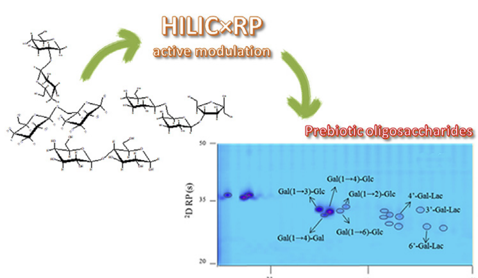
HIGHLIGHTS

- Active modulation used to carry out the HILIC × RP analysis of oligosaccharides.
- Commercial prebiotic oligosaccharides involving diverse glycosidic linkages are analyzed.
- ABEE-derivatized di- and trisaccharides are separated using BEH-amide and C18 columns.
- First reported LC × LC methodology applied to carbohydrates analysis.
- The method opens new perspectives in the field of carbohydrates analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbohydrates are one of the most important ingredients in foods. They are normally present as complex mixtures with different glycosidic linkages, monomeric units and degrees of polymerization. This structural heterogeneity impairs their comprehensive characterization and requires the use of analytical techniques with high resolving power and sensitivity. The use of chromatographic techniques, especially liquid chromatography (LC), has been extremely helpful for the analysis of carbohydrates. However, in many cases, the use of monodimensional LC is not enough to resolve these complex mixtures; then, the use of techniques with a higher resolving power, as multidimensional LC, could be a good alternative. To the best of our knowledge, our findings are pioneer in applying online LC × LC for the analysis of carbohydrate mixtures. For this purpose, different conditions such as stationary phases (BEH amide, C_{18} and PGC columns) and chromatographic conditions for the separation of di- and trisaccharide mixtures were optimized. The BEH amide × C_{18} combination was selected for the LC × LC analysis of carbohydrate standards with different degrees of polymerization, linkages and monomeric units. In order to allow their proper UV detection, carbohydrates were previously derivatized using *p*-aminobenzoic ethyl ester. This method also resulted to be successful for the separation of commercial prebiotic mixtures of galacto-oligosaccharides and gentio-oligosaccharides. This is the first time that LC × LC has been applied for the separation of bioactive carbohydrate mixtures and it could be considered as a powerful analytical technique for the characterization of other oligosaccharide complex mixtures.

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1. Introduction

Carbohydrates are one of the most important food constituents, usually present as complex mixtures of isomeric molecules of different degrees of polymerization which only differ in the configuration of their hydroxyl groups and in the position of their glycosidic linkages. The primary role of food carbohydrates is to provide energy to all cells in the body and dietary fiber [1]. Within dietary fiber, non-digestible carbohydrates, which are not absorbed in the small intestine and, therefore, move down to be fermented in the large intestine, have drawn attention because their beneficial impact on human health. Concretely, low glycemic index foods, whose intake is related to a reduced risk of common Western chronic diseases associated with central obesity and insulin resistance [2,3], are characterized by the presence of non-digestible or slow-absorption carbohydrates instead of free sugars. Those non-digestible carbohydrates having the ability to be "selectively utilized by living host microorganisms (usually, in the gut microbiota ecosystem) conferring a health benefit" are defined as prebiotics [4]. Among the carbohydrates with a well-recognized prebiotic status, galactooligosaccharides (GOS) are by far the most complex structurally. They may comprise a wide mixture of oligosaccharides that can vary from 1 to 8 galactose (Gal) units and a terminal glucose (Glc) linked by a great diversity of β-glycosidic linkages, mainly $(1 \rightarrow 3)$, $(1 \rightarrow 4)$ and/or $(1 \rightarrow 6)$. The presence of $(1 \leftrightarrow 1)$ and $(1 \rightarrow 2)$ linkages has also been reported for commercial dietary GOS [5]. Similarly, other potential prebiotic carbohydrates such as gentiooligosaccharides (GEOS), xylooligosaccharides, isomaltooligosaccharides, etc. are also complex structures. For this reason, the analysis and structural characterization of prebiotic carbohydrates, which is required to fully understand their functionality and correlation with the chemical structure, is a challenging task.

Nowadays, high performance liquid chromatography (LC), combined with pulsed amperometric, refractive index, ultraviolet and fluorescence detectors or mass spectrometry, is the most used chromatographic technique for the analysis of complex mixtures of oligosaccharides. This is due to the huge development during the last decades of a wide range of new support materials and/or stationary phases operating under different separation modes [6]. Derivatization procedures of oligosaccharides before their LC separation have also been suggested to improve their chromatographic properties [7,8]. Nevertheless, there are still important limitations for the efficient separation of complex oligosaccharide mixtures when several structural features, such as degree of polymerization, linkage pattern, monomeric composition and/or isomerism, differ. In this context, the application of multidimensional liquid chromatography and, particularly, the use of on-line comprehensive two-dimensional LC (LC × LC) could be a very powerful and promising alternative to overcome the drawbacks on the capability of separating complex oligosaccharide mixtures by the currently available monodimensional LC (1DLC) methods. Nevertheless, although the applications developed by on-line LC × LC devoted to food and natural products are increasing [9–11], to the best of our knowledge there is a remarkable lack of comprehensive LC applications for carbohydrates analysis. Difficulties in finding the correct columns combinations to attain good degrees of orthogonality between dimensions, solvent mismatch problems during fraction transfer in on-line methods, as well as complications related to detection are partly responsible for this absence of LC × LC methods.

Therefore, considering these analytical challenges, the current work aims to develop a novel LC × LC method for the efficient separation of di- and trisaccharide mixtures with different glycosidic linkages and monomeric composition. A thorough optimization by using a wide range of carbohydrate standards was previously accomplished by 1DLC to select the most appropriate stationary phase combination for the LC × LC separation as well as the derivatization procedure to facilitate their UV-based detection. The optimized method was applied to the separation of different prebiotic oligosaccharide mixtures, dominated by di- and trisaccharides, such as three commercial dietary GOS differing in the predominant β-glycosidic linkage and a sample of GEOS. This latter product is a novel functional oligosaccharide mixture composed of glucose units mainly linked through $\beta(1 \rightarrow 6)$ glycosidic bonds.

2. Materials and methods

2.1. Standards and oligosaccharide mixtures

1,3-Galactobiose (α-Gal-$[1 \rightarrow 3]$-Gal), 1,4-galactobiose (β-Gal-$[1 \rightarrow 4]$-Gal), 1,6-galactobiose (β-Gal-$[1 \rightarrow 6]$-Gal), galactotriose (α-Gal-$[1 \rightarrow 3]$-β-Gal-$[1 \rightarrow 4]$-Gal) were acquired from Dextra Laboratories (Reading, UK), whereas kojibiose (α-Glc-$[1 \rightarrow 2]$-Glc), kojitriose (α-Glc-$[1 \rightarrow 2]_2$-Glc), lactose (β-Gal-$[1 \rightarrow 4]$-Glc), laminaribiose (β-Glc-$[1 \rightarrow 3]$-Glc), laminaritriose (β-Glc-$[1 \rightarrow 3]_2$-Glc), isomaltose (α-Glc-$[1 \rightarrow 6]$-Glc), isomaltotriose (α-Glc-$[1 \rightarrow 6]_2$-Glc), maltose (α-Glc-$[1 \rightarrow 4]$-Glc), maltotriose (α-Glc-$[1 \rightarrow 4])_2$-Glc), cellobiose (β-Glc-$[1 \rightarrow 4]$-Glc), were obtained from Sigma (St. Louis, USA). 3'-Galactosyl lactose (β-Gal-$[1 \rightarrow 3]$-β-Gal-$[1 \rightarrow 4]$-Glc), 4'-Galactosyl lactose (β-Gal-$[1 \rightarrow 4]$-β-Gal-$[1 \rightarrow 4]$-Glc) and 6'-Galactosyl lactose (β-Gal-$[1 \rightarrow 6]$-β-Gal-$[1 \rightarrow 4]$-Glc) were acquired from Carbosynth (Berkshire, UK). *p*-Aminobenzoic ethyl ester (ABBE) was obtained from Sigma (St. Louis, USA).

Vivinal-GOS (GOS1) was kindly provided by Friesland Foods Domo (Zwolle, The Netherlands); BiMuno (Clasado, Reading, UK) (GOS2) and PromoVita (Dairy Crest, Esher, UK) (GOS3) were acquired in local markets. Gentio-oligosaccharides (GEOS) were acquired from Wako Chemicals GmbH (Neuss, Germany).

Commercial standards and commercial oligosaccharide mixtures were dissolved in the corresponding HPLC mobile phase at a concentration of 0.1 mg mL^{-1} and filtered through a nylon FH membrane (0.22 μm) (Millipore, Bedford, MA, USA) before injection. All HPLC analyses were carried out in duplicate.

2.2. Derivatization procedure

The absence of chromophore groups in carbohydrates, which hinder their direct detection by UV, was solved by a previously developed derivatization procedure with *p*-aminobenzoic ethyl ester (ABBE) [12]. Briefly, the ABEE reagent was firstly prepared by mixing 165 mg ABEE, 35 mg sodium cyanoborohydride, 41 μL acetic acid, and 0.35 mL warm methanol. Then, 40 μL of ABEE reagent was added to 10 μL sample (dissolved in water) and kept at 80 °C for 1 h. After cooling, 0.2 mL of H_20 and 0.2 mL of chloroform were added. The sample was then centrifuged for 1 min and the upper aqueous layer recovered and used for analysis. These derivatives were stable at least for 1 week (RSD 3.4%).

2.3. One-dimensional (1DLC) analysis of carbohydrates

2.3.1. Instrumentation

Conventional LC analyses were carried out on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) equipped with an oven (Kariba Instruments, UK) and a UV detector (Agilent) with a wavelength set at 304 nm.

2.3.2. Separation conditions

Three different columns based on different LC operation modes were evaluated. These columns were an ethylene bridge hybrid with trifunctionally-bonded amide phase (BEH amide column) (150 × 4.6 mm; 3.5 μm) from Waters (Hertfordshire, UK), a C_{18} column (Zorbax Eclipse XDB) (50 × 4.6 mm; 1.8 μm) from Agilent Technologies (Waldbronn, Germany) and a Hypercarb porous graphitic carbon (PGC) column (150 × 2.1 mm; 3 μm) from Thermo Scientific (Waltham, MA, USA). Different gradients of ACN and water in different proportions, flow rates and analysis times were assayed for the separation of the derivatized carbohydrates for each evaluated column, obtaining the following optimum gradient conditions:

BEH amide stationary phase: Acetonitrile (A) and water (B) eluted using the following gradient: 0 min, 15% B; 4 min, 15% B; 14 min, 40% B; 19 min, 40% B.

C_{18} column: Water (A) and acetonitrile (B) eluted using the following gradient: 0 min, 5% B; 30 min, 40% B; 31 min, 60% B; 36 min, 60% B.

Porous graphitic carbon (PGC) column: Water (A) and acetonitrile (B) eluted using the following gradient: 0 min, 60% B; 20 min, 90% B; 21 min, 90% B.

For all of them 10 min was used as equilibration time, a flow rate of 0.4 mL min^{-1} was selected and the injection volume was 5 μL.

Different chromatographic parameters were considered for optimization of 1DLC methods, namely, retention time (t_R), peak width at half height (w_h), symmetry factor (S), as the ratio of the front half to back half widths at 10% of the peak height, and resolution (R_S), calculated according to

$$R_s = 2\frac{(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})} \quad (1)$$

where sub-indexes 1 and 2 refer to two consecutive eluting carbohydrates, and w_b is the peak width at baseline.

2.4. LC × LC analysis of carbohydrates

2.4.1. Instrumentation

Comprehensive two-dimensional liquid chromatography instrumentation consisted on a first dimension (^{1}D) composed of an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a diode array detector which was connected at the exit of the second dimension. The second dimension (^{2}D) was carried out using an additional LC pump (Agilent 1290 Infinity). An electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) was used as modulator to connect both dimensions. Two identical sampling loops (50 or 80 μL) or two C_{18} trapping columns (10 × 3 mm, 2.6 μm, Accucore, Thermo Scientific, Waltham, MA, USA) were connected to the switching valve to collect the fractions from the ^{1}D and to inject them into the ^{2}D. Separations were recorded at 304 nm at the maximum available sampling rate (20 Hz). For the active modulation configuration, an additional make-up flow was provided by a third LC pump (Agilent 1200 series) connected through a T-piece between the outlet of ^{1}D and the switching valve. LC image software (Zoex Corp., Houston, TX, USA) was used to plot the results as 2D and 3D images.

2.4.2. LC × LC separation conditions

A HILIC separation in the ^{1}D was coupled to a reversed phase separation in the ^{2}D. After optimization (see Sections 3.1 and 3.2), the following final conditions were employed:

^{1}D separation: A BEH amide column (150 × 2.1 mm; 3.5 μm, Waters, Hertfordshire, UK) was employed at a flow rate of 0.05 mL min^{-1}. Under optimum conditions, H_2O (A) and ACN (B) were used as mobiles phases, eluted using the following gradient: 0 min, 90% B, 60 min 50% B; 70 min, 50% B; 71 min, 90% B; 85 min 90% B.

^{2}D separation: two columns with different length (partially-porous C_{18} Ascentis Express 30 or 50 × 4.6 mm, 2.7 μm, Supelco, Bellefonte, CA, USA) were tested. Gradient, flow rate, temperature, type of acid and solvents used were optimized separately. The optimum conditions were achieved using the column of 50 mm length and 2.7 μm particle size with H_2O (solvent A) and ACN (solvent B) as mobile phases. The optimum gradient was as follow: 0 min, 10% B; 0.75 min, 40% B; 0.76 min, 90% B; 0.9 min, 10% B; and re-equilibrium at starting conditions until 1 min. The flow rate was set at 2.5 mL min^{-1}.

Once these parameters were optimized, the coupling between both dimensions was achieved employing 1 min repetitive ^{2}D separations; thus, the modulation time was 1 min. The third pump used to achieve the active modulation was set at 500 μL min^{-1}, delivering solvent with the same composition than the initial ^{2}D gradient conditions (10% B).

2.5. Calculations

To study the performance of the developed methods, the attainable peak capacity and orthogonality were calculated. Individual peak capacities in each dimension were calculated according to eq. (2) [13] as follows:

$$n_c = 1 + \frac{t_g}{\left(\frac{1}{n}\right)\sum_1^n w} \quad (2)$$

Where, n_c represents the peak capacity, n is the number of compounds, t_g is the gradient time and, w is the peak width. With the individual peak capacity values of each dimension, the total theoretical peak capacity of the two-dimensional method was calculated as,

$$^{2D}n_c = {}^1n_c \times {}^2n_c \quad (3)$$

This value is clearly overestimated since it does not consider the effect of undersampling of the ^{1}D and the peak broadening effect occurred during modulation. To solve this problem, the effective peak capacity was calculated according to eq. (4) [14]:

$$^{2D}n'_c = \frac{^1n_c \times {}^2n_c}{\sqrt{1 + 3.35 \times \left(\frac{^2t_c\,{}^1n_c}{^1t_G}\right)^2}} \quad (4)$$

In this case, $^{2D}n'_c$ is the effective 2D peak capacity, ^2t_c is the second dimension cycle time and ^1t_G is the first dimension gradient time. Lastly, the corrected peak capacity was defined as:

$$^{2D}n_{c,corrected} = {}^{2D}n'_c \times A_0 \quad (5)$$

Where A_0 is the orthogonality value calculated in agreement with the asterisk equations method [15].

3. Results and discussion

3.1. 1DLC analysis of di- and trisaccharides

Preliminary experiments in 1DLC were carried out to select the most appropriate stationary phase combination for further LC × LC separation of ABEE oligosaccharide mixtures. Several di- and trisaccharide standards with different monomeric units and glycosidic linkages were selected for the optimization of the separation in each stationary phase. Three columns based on different HPLC operation mechanisms were assayed. In all cases, chromatographic conditions were optimized in terms of the shortest t_R, the best peak symmetry, the highest R_s and the lowest w_h, using acetonitrile and water as mobile phases.

As it can be observed in Table 1, on BEH amide stationary phase, ABEE disaccharides eluted at shorter retention times than trisaccharides, with an excellent resolution between them. For instance, a resolution of $R_s = 2.6$ between the last disaccharide (1,6-galactobiose) and the first trisaccharide to elute (laminaritriose) was achieved. Similar results had been previously observed by Brokl et al. [16] and Hernández-Hernández et al. [17] for non-derivatized carbohydrates, who found that this column was useful for the separation of oligosaccharides with different degree of polymerization. However, a poor resolution ($R_s < 0.48$) among the different disaccharides was obtained, except for kojibiose and 1,6-galactobiose and for maltose and 1,4-galactobiose. On the contrary, trisaccharides were, in general, appropriately resolved ($R_s > 0.90$), except for galactotriose and 3'-galactosyl-lactose and for isomaltotriose and kojitriose. For a given glycosidic linkage, di- and trisaccharides composed by glucosyl units eluted before those containing galactosyl units (e.g., laminaribiose and 1,3-galactobiose; maltose and 1,4-galactobiose; isomaltose and 1,6-galactobiose; maltotriose and 4'-galactosyl-lactose). For each polymerization degree, in general, glucosyl-di- and trisaccharides with 1 → 3 glycosidic linkages eluted first, while the most retained compounds were those with 1 → 6 linkages. A similar behavior was observed for galactosyl-di- and trisaccharides, although isomers with 1 → 4 linkages were the first to elute.

Table 1
Retention time (t_R, min), peak width (w_h), resolution (R_s) and symmetry (S) of standard carbohydrates analyzed using a BEH amide, a C_{18} and a PGC column.

Column	Compound	t_R	w_h	S	R_s
BEH amide	Laminaribiose	14.00	0.36	1.16	0.04
	Cellobiose	14.03	0.34	1.20	0.22
	Maltose	14.16	0.37	1.48	1.04
	1,4-Galactobiose	14.80	0.35	1.95	0.08
	Lactose	14.84	0.27	1.18	0.48
	Isomaltose	15.09	0.32	1.41	0.01
	1,3-Galactobiose	15.09	0.34	1.73	0.16
	Kojibiose	15.17	0.25	1.02	1.39
	1,6-Galactobiose	15.77	0.25	1.08	2.60
	Laminaritriose	16.79	0.21	1.47	1.31
	Maltotriose	17.26	0.21	0.91	1.32
	4'- Galactosyl lactose	17.71	0.19	1.55	0.94
	Galactotriose	18.02	0.19	0.98	0.67
	3'-Galactosyl lactose	18.24	0.19	1.27	0.90
	Isomaltotriose	18.53	0.19	1.21	0.28
	Kojitriose	18.62	0.17	0.79	1.38
	6'-Galactosyl lactose	19.01	0.16	0.81	
C_{18}	6'-Galactosyl lactose	10.70	0.19	0.38	5.41
	Kojitriose	12.03	0.10	0.84	3.32
	Galactotriose	12.63	0.11	0.85	2.25
	4'-Galactosyl lactose	13.06	0.11	0.88	1.37
	Isomaltotriose	13.32	0.11	0.86	1.59
	Kojibiose	13.63	0.11	0.86	0.44
	1,4-Galactobiose	13.71	0.11	0.86	0.11
	3'-Galactosyl lactose	13.73	0.11	0.84	1.38
	1,3-Galactobiose	14.00	0.11	0.86	0.21
	Lactose	14.04	0.12	0.86	0.62
	Isomaltose	14.15	0.11	0.91	1.11
	Laminaritriose	14.36	0.12	0.83	0.08
	Laminaribiose	14.38	0.12	0.82	0.69
	Cellobiose	14.51	0.11	0.86	1.09
	Maltotriose	14.72	0.11	0.86	0.56
	1,6-Galactobiose	14.83	0.12	0.87	1.71
	Maltose	15.17	0.11	0.87	
PGC	Cellobiose	3.23	0.22	0.41	0.75
	1,4-Galactobiose	3.50	0.20	0.38	0.36
	Isomaltose	3.66	0.31	0.51	0.20
	Lactose	3.76	0.28	0.28	0.05
	Laminaribiose	3.78	0.33	0.43	0.14
	1,6-Galactobiose	3.87	0.35	0.53	0.08
	1,3-Galactobiose	3.91	0.34	0.25	0.24
	Kojibiose	4.04	0.27	0.31	0.87
	4'-Galactosyl lactose	4.42	0.25	0.38	0.35
	Laminaritriose	4.59	0.29	0.34	0.27
	Isomaltotriose	4.72	0.31	0.37	0.56
	Kojitriose	5.02	0.31	0.32	0.18
	3'-Galactosyl lactose	5.11	0.31	0.34	0.18
	6'-Galactosyl lactose	5.21	0.33	0.32	0.05
	Galactotriose	5.23	0.26	0.39	0.99
	Maltose	5.77	0.38	0.37	2.96
	Maltotriose	7.75	0.41	0.36	

In general, good peak widths (w_h: 0.16–0.21 min) and symmetry values (0.79–1.55) were observed for trisaccharides that eluted under the optimized conditions in BEH amide stationary phase. Poorer results were obtained for disaccharides (w_h: 0.25–0.37 min and symmetry: 1.02–1.95).

Regarding C_{18} stationary phase, the separation of ABBE oligosaccharides was not size-dependent (Table 1), as it had been previously observed for oligosaccharides released from glycosphingolipids [12]. Trisaccharides eluted in the range 10.70 min (6'-galactosyl-lactose) – 14.72 min (maltotriose), while disaccharides eluted between 13.63 min (kojibiose) and 15.17 min (maltose). Contrary to that observed in the BEH amide column, some trisaccharides eluted before disaccharides. The elution order was neither associated to the composition of monomeric units (Glc and Gal) nor to the glycosidic linkages. This column provided good w_h values (0.11–0.19 min) for all the carbohydrates. In general, resolution values were acceptable for some carbohydrates although several coelutions were observed for both di- and trisaccharides (e.g., laminaribiose and laminaritriose). Peaks also showed a satisfactory symmetry (0.8–0.9), except for 6'-galactosyl-lactose (0.38).

Graphitized carbon emerged as stationary phases in LC as an alternative to RP columns for the analysis of polar compounds [18] and it has been successfully used for the separation of neutral oligosaccharides, *N*-linked-oligosaccharides or chito-oligosaccharides, providing good resolution for non-derivatized carbohydrates [19]. In general, ABEE carbohydrates showed low retention times under optimal chromatographic conditions in PGC column (Table 1). Most disaccharides eluted earlier than trisaccharides; only maltose (5.77 min) exhibited a relatively high retention in this stationary phase, eluting at the end of the chromatogram, surpassed only by maltotriose (7.75 min). Broad peaks (from 0.20 to 0.41 min) with poor symmetry (0.25–0.53) were obtained. Regarding R_s, only galactotriose, maltose and maltotriose were well resolved (R_s,>0.99).

Retention times of ABEE di- and trisaccharides eluting on the three evaluated columns were reconstructed in two dimensions (see Fig. S1). As can be observed, the combination of C_{18} column with both, BEH amide and PGC columns provided the best separation of target carbohydrates. In both combinations, coelutions occurred in one dimension could theoretically be resolved in the second dimension. However, considering the peak width and symmetry values commented before, the use of the BEH amide column in the ^{1}D and a C_{18} column in the ^{2}D was considered, in theory, the most promising alternative for LC × LC separation of oligosaccharide mixtures.

3.2. Optimization of the LC × LC separation of oligosaccharides

To find appropriate chromatographic conditions compatible with LC × LC for the separation of carbohydrates is a great challenge, mainly considering the broad distribution of the compounds in the ^{1}D available separation space sought, as well as the limited separation time afforded in the ^{2}D. As a result, different prerequisites were imposed to the separation conditions for optimization; in the ^{1}D, the attainment of good separations with a maximum flow rate of 0.05 mL min^{-1} was targeted, whereas proper individual separations of less than 1 min were aimed in the ^{2}D.

Starting from the mobile phases already selected in conventional LC (acetonitrile and water), the gradient profile was adapted to the flow rate reduction and subsequently optimized. Fig. S2 shows a comparison between the different chromatograms obtained during the optimization study of the ^{1}D separation using commercial disaccharide and trisaccharide standards with different linkages and monomeric units. The best separation of target carbohydrates was achieved varying acetonitrile from 90% to 50% in 60 min. As it is known, under HILIC conditions, the sample solvent has a strong influence on the attainable separation, which is even greater in the case of narrow bore columns as the one used in the present work. A high water content in the sample may break the interaction between analytes and the aqueous layer surrounding the stationary phase particles hampering the separation due to lack of retention. To avoid this problem, the derivatized samples were diluted with acetonitrile before injection.

On the other hand, two ^{2}D C_{18} columns with diverse length (30 and 50 mm) were tested. Acetonitrile and water were also chosen as mobile phases under RP conditions. For each column, different flow rates (1, 2 and 2.5 mL min^{-1}) and gradients were studied with the aim to obtain a good separation within the previously allotted time (1 min). Optimum conditions involved the use of 2.5 mL min^{-1} as flow rate using the following gradient: 0 min, 10% B; 0.75 min, 40% B; 0.76 min, 90% B; 0.9 min, 10% B; and re-equilibrium at starting conditions until 1 min. Chromatographic profiles of a disaccharide mixture obtained at different flow rates under the selected optimum gradient conditions for ^{2}D are shown in Fig. S3. Under those conditions, both tested columns provided very similar performance (Figs. S3D and E). Considering that the separation was finished in less than 1 min in both cases, the 50 mm length column was selected for further LC × LC optimization due to its longer separation space available.

Once the individual conditions for each dimension compatible with LC × LC were determined, the overall optimization of the two-dimensional separation was carried out. The coupling between the two dimensions was established by using two 50 μL identical sampling loops installed in the switching valve. This internal volume was the smallest needed to collect each complete fraction eluting from the ^{1}D during the modulation period. Under those conditions, the analysis of a di- and trisaccharide mixture was carried out. Results obtained are shown in Fig. 1A. Although some separation was observed, peaks were grouped in two areas of the 2D plot corresponding to the beginning and half of each ^{2}D analysis, suggesting that no proper retention/elution was obtained. The lack of retention (peaks doubled) was most probably related to solvent strength incompatibility between dimensions. In fact, the fraction solvent was by far stronger for the ^{2}D than the initial gradient conditions. This problem has been widely reported in HILIC × RP couplings [20]. In other applications, an increase of the fraction volume was shown to be positive towards the elimination of this issue; the use of sampling loops with internal volume larger than strictly needed to accommodate the ^{1}D fraction was demonstrated to be useful to produce a dilution effect with ^{2}D compatible solvent [21]. Consequently, this approach could be effective in reducing the overall fraction solvent strength and, thus, improving the attainable separation. To test this potential solution, the same separation conditions were applied increasing the sampling loops internal volume to 80 μL. As it can be observed in Fig. 1B, no noticeable improvement was produced.

The next step carried out to overcome this problem was the use of trapping columns in the interface instead of sampling loops, looking for a focusing modulation able to increase analyte retention. C_{18} trapping columns were employed using the same 2D analytical conditions (Fig. 1C). The selection of the stationary material was made in order to match the selectivity used in the ^{2}D. The two tested trapping columns possessed a void volume of 50 μL, thus, capable of collecting the whole fraction coming from the ^{1}D and were eluted in forward elution mode. However, under the tested conditions, the problem persisted and no complete retention was obtained using this focusing modulation approach.

For this reason, the use of active modulation was investigated in this application. The ability of active modulation to resolve solvent strength mismatch problems between dimensions has been previously demonstrated [20–22]. The same trapping columns previously installed were maintained but an additional make-up flow was introduced at the exit of the ^{1}D and mixed with the fraction eluting from the ^{1}D column and directed to the modulator. The make-up flow composition was selected to match the initial conditions found in the ^{2}D gradient (90:10 water/acetonitrile), whereas the flow rate was set according to our previous experience as 10-times the ^{1}D flow rate, i.e., 0.5 mL min^{-1} [20]. The rest of separation conditions were maintained. As can be seen in Fig. 1D, the use of active modulation resolved the retention issue observed in the other set-ups. In this case, the separation in the ^{2}D was improved, and no doubled peaks were observed. Thus, in spite of a more complicated set-up, including an additional pump for the make-up flow, this strategy clearly permitted to produce a concentration step in the trapping columns thanks to the dilution on a fully compatible solvent composition. Once the fraction collection was finished, the ^{2}D gradient started and the compounds were eluted from the trapping column accordingly. These separation conditions were, therefore, selected for further analysis.

3.3. Analysis of oligosaccharides by LC × LC

The optimized separation method was applied to the analysis of different di- and trisaccharide mixtures with different glycosidic linkages (1 → 2, 1 → 3, 1 → 4 and 1 → 6 bonds) and monomeric units (Glc and Gal). Fig. 2 shows the LC × LC contour plots of the mixtures of glucosyl-disaccharides (panel A, kojibiose, laminaribiose, maltose, cellobiose and gentiobiose), glucosyl-trisaccharides (panel B, kojitriose, laminaritriose, maltotriose and isomaltotriose), galactosyl-disaccharides (panel C, 1,3-galactobiose, 1,4-galactobiose, lactose and 1,6-galactobiose) and galactosyl-trisaccharides (panel D, galactotriose, 3'-galactosyl lactose, 4'-galactosyl lactose and 6'-galactosyl lactose). These mixtures were chromatographically resolved in the two-dimensions while some of these compounds coeluted in monodimensional LC. This is the case, for instance, of glucosyl-disaccharides (Fig. 2A): kojibiose (with α1→2 linkage) was separated from isomaltose (with α1→6 linkage) and cellobiose (with β1→4 linkage) from laminaribiose (with β1→3 linkage) in the ^{2}D, while maltose (with α1→4 linkage) was separated from cellobiose in ^{1}D. A similar pattern was also observed for their corresponding glucosyl-trisaccharides (having the same glycosidic linkages and monomeric composition; Fig. 2B). The formation of group-type patterns on the two-dimensional plane has been previously observed for other type of compounds in LC × LC [10]. It has been seen that regular variation of the compound structures could lead to ordering in a chromatogram. Then, this method can be useful for the identification of unknown carbohydrates with a certain degree of structural similarities present in complex samples.

Regarding galactosyl-di- and trisaccharides (Fig. 2C and D,

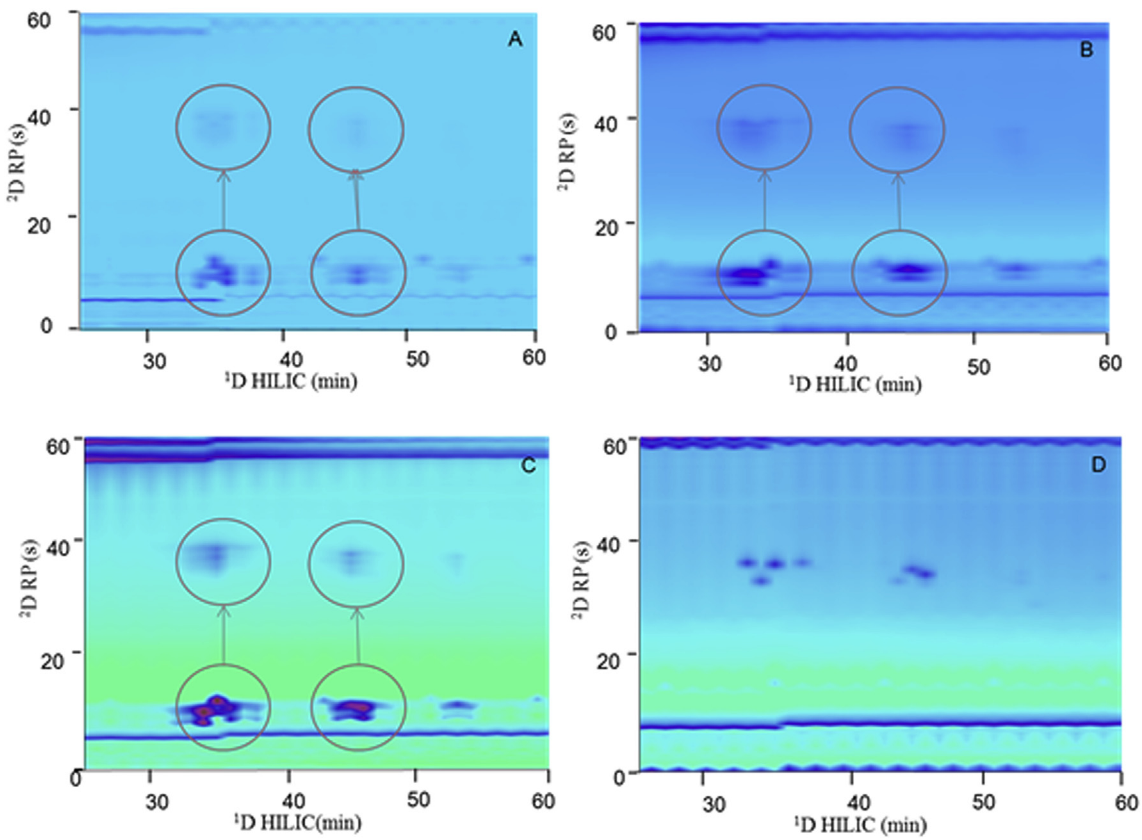

Fig. 1. 2D HILIC × RP separation of a di- and trisaccharide mixture using: A, non-focusing modulation with 50 µL sampling loops; B, non-focusing modulation with 80 µL sampling loops; C, focusing modulation using two trapping columns (C_{18}, 10 × 3 mm, 2.6 µm); D, focusing modulation with trapping columns and active modulation. For detailed experimental conditions, see text.

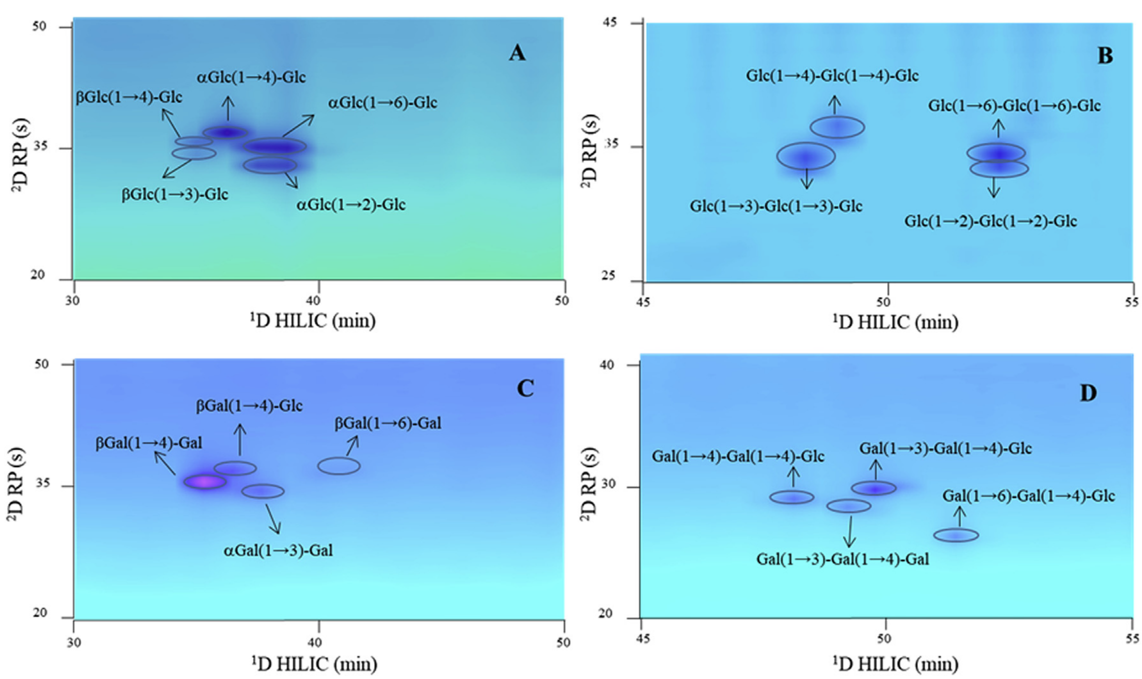

Fig. 2. 2D plots (304 nm) obtained under optimum HILIC × RP separation conditions for different di- (A and C) and trisaccharide (B and D) mixtures. For detailed analytical conditions, see text.

respectively), a good separation was observed for each mixture; however, a group-type pattern could not be established due to the different linkages and monomeric units constituting these carbohydrates.

Then, to evaluate the advantages of the new LC × LC method for the separation of di- and trisaccharides with different glycosidic linkages and monomeric units, three commercial prebiotic mixtures of GOS and one commercial preparation of GEOS were analyzed. The 2D plots obtained for these samples are shown in Fig. 3. Identification of the different di- and trisaccharides was considered tentative and it was carried out based on the comparison of retention times in ^{1}D and ^{2}D of the target carbohydrates with those of the corresponding standards, when available, and/or data from literature regarding GOS composition [17,23]. Carbohydrates were properly resolved using the developed LC × LC method despite the usual differences in concentration levels of target carbohydrates present in real samples. This was the case, for example, for the structurally different galactosyl-lactoses (4', 3' and 6') present in GOS1 sample at dissimilar concentrations and which were efficiently resolved (Fig. 3). Furthermore, the optimized LC × LC method provided the benefit of resolving isomeric oligosaccharides respect to 1DLC in all assessed samples. For instance, 4'-galactosyl-lactose was efficiently resolved in GOS1 and GOS3 samples by LC × LC separation from an unknown trisaccharide which coeluted by 1DLC (Fig. 3). This was also the case for the separation of β-galactosyl-galactoses from β-galactosyl-glucoses observed in GOS2 and GOS3 samples, as well as among the complex mixture of glucobioses present in the GEOS sample (Fig. 3).

To establish a comparison of the method performance for the different samples analyzed, peak capacities and orthogonality values were estimated in each case (Table 2). As it can be observed, high effective peak capacity values, up to 1425 when under-sampling from the ^{1}D was already considered, were obtained for all the studied samples. These values demonstrate theoretically the high separation power obtained using the optimized methodology. However, more realistic peak capacity figures can be offered derived from the calculation of the orthogonality degree. To do that, the orthogonality degree of each separation was also estimated. In this case, relatively good values around 40% were obtained for all the samples except GOS3. In general, good use of the available 2D space was obtained, considering that the separated peaks were not grouped in the diagonal, as it often happens with not entirely non-correlated separation mechanisms. However, the ^{2}D separation space was not completely used, as peaks tended to elute around a 20 s window in spite of the longer gradient employed. This fact is the main responsible for not attaining even higher A_0 values, and clearly illustrates some of the challenges behind carbohydrates analysis. In any case, when the effective peak capacity values were corrected by orthogonality following eq. (5), good values were obtained for GOS1, GOS2 and GEOS of up to 531, which are comparable to other corrected peak capacities in other successful applications [20].

Table 2
Peak capacity and orthogonality values obtained for the 4 commercial prebiotic oligosaccharide products analyzed using the optimum HILIC × RP conditions determined.

	GOS1	GOS2	GOS3	GEOS
^{1}D peak capacity, ^1n_c	42	38	41	37
^{2}D peak capacity, ^2n_c	62	54	48	64
Theoretical peak capacity, $^{2D}n_c$	2604	2052	1968	2368
Effective peak capacity, $^{2D}n'_c$	1425	1176	1084	1370
Corrected peak capacity, $^{2D}n_{c,corr}$	457	454	103	531
Orthogonality, A_0	32%	39%	10%	39%

A_O, orthogonality; $^{2D}n_c = {}^1n_c \times {}^2n_c$; $^{2D}n'_c$: calculated according to Ref. [13]; $^{2D}n_{c\ corr.}$: $^{2D}n'_c \times A_O$.

4. Conclusions

A new HILIC × RP method has been developed for the separation of ABEE derivatized di- and trisaccharides with different glycosidic linkages and monomeric units. After preliminary analyses by 1DLC, the column combination involving the use of BEH amide column in the ^{1}D and C_{18} in the ^{2}D was selected for the separation of these components by LC × LC. Active modulation was shown to be useful to solve the solvent mismatch problems encountered during the

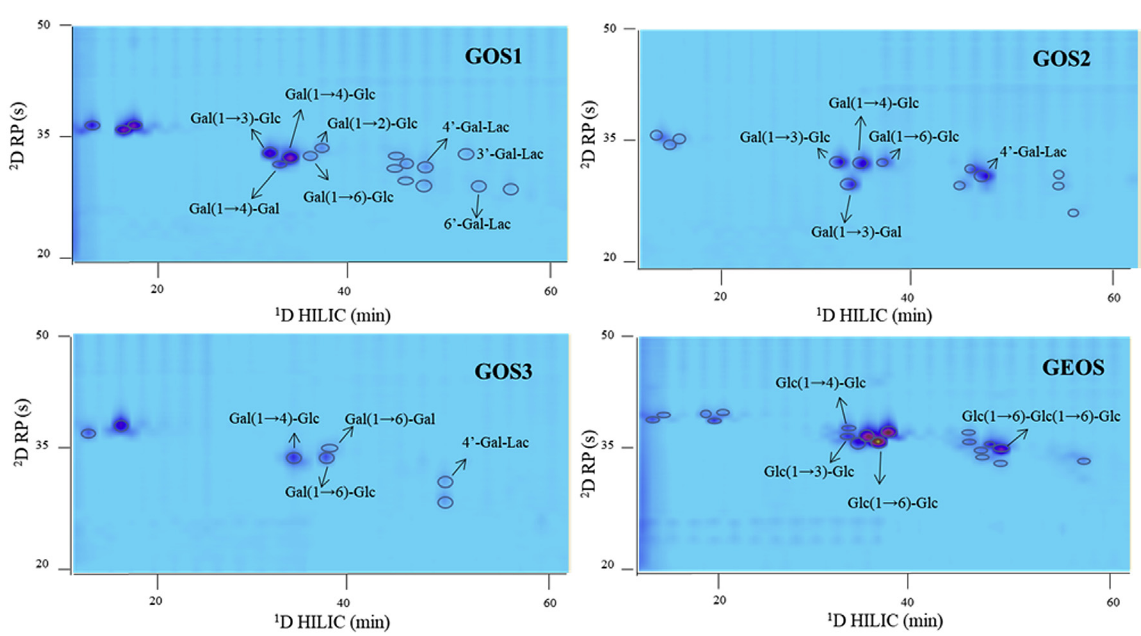

Fig. 3. 2D plots (304 nm) obtained under optimum HILIC × RP separation conditions for different commercial prebiotic oligosaccharide products. For detailed analytical conditions, see text.

transfer to the ^{2}D. Thanks to the optimized methodology, it was possible to analyze, for the first time, commercial prebiotic oligosaccharide mixtures of different nature (GOS and GEOS) involving the presence of diverse glycosidic linkages that were not separated by conventional LC. Our data demonstrate the utility of on-line LC $\times$ LC to analyze complex oligosaccharide mixtures of low degree of polymerization, and open the door to further applications in the field of carbohydrates analysis.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.01.040.

References

[1] J. Slavin, J. Carlson, Carbohydrates, Adv Nutr 5 (2014) 760–761.
[2] D.J.A. Jenkins, C.W.C. Kendall, L.S.A. Augustin, S. Franceschi, M. Hamidi, A. Marchie, A.L. Jenkins, M. Axelsen, Glycemic index: overview of implications in health and disease, Am. J. Clin. Nutr. 76 (1) (2002), 266S-73S.
[3] L.S. Augustin, S. Franceschi, D.J.A. Jenkins, C.W.C. Kendall, C. La Vecchia, Glycemic index in chronic disease: a review, Eur. J. Clin. Nutr. 56 (11) (2002) 1049–1071.
[4] G. Gibson, R. Hutkins, M.E. Sanders, S.L. Prescott, R.A. Reimer, S.J. Salminen, K. Scott, C. Stanton, K.S. Swanson, P.D. Cani, K. Verbeke, G. Reid, The international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics, Nat. Rev. Gastroenterol. Hepatol. 14 (2017) 491–502.
[5] S.S. van Leeuwen, B.J. Kuipers, L. Dijkhuizen, J.P. Kamerling, Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products, Carbohydr. Res. 425 (2016) 48–58.
[6] F.J. Moreno, A. Montilla, M. Villamiel, N. Corzo, A. Olano, Analysis, structural characterization, and bioactivity of oligosaccharides derived from lactose, Electrophoresis 35 (2014) 1519–1534.
[7] S. Bank, P. Kapkova, Liquid chromatography-Mass spectrometry of carbohydrates derivatized with biotinamidocaproyl hydrazide, Carbohydrates (2012) 55–80.
[8] M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis, J. Chromatogr. A 1218 (2011) 118–123.
[9] S. Ji, S. Wang, H. Xu, Z. Su, D. Tang, X. Qiao, M. Ye, The application of on-line two-dimensional liquid chromatography (2DLC) in the chemical analysis of herbal medicines, J Pharmaceut Biomed 160 (2018) 301–313.
[10] F. Cacciola, S. Farnetti, P. Dugo, P.J. Marriott, L. Mondello, Comprehensive two-dimensional liquid chromatography for polyphenol analysis in foodstuffs, J. Separ. Sci. 40 (1) (2017) 7–24.
[11] P.Q. Tranchida, P. Donato, F. Cacciola, M. Beccaria, P. Dugo, L. Mondello, Potential of comprehensive chromatography in food analysis, TrAC Trends Anal. Chem. (Reference Ed.) 52 (2013) 186–205.
[12] H. Kwon, J. Kim, Determination in monosaccharides in glycoproteins by reverse-phase high-performance liquid chromatography, Anal. Biochem. 215 (1993) 243–252.
[13] U.D. Neue, Theory of peak capacity in gradient elution, J. Chromatogr. A 1079 (1–2) (2005) 153–161.
[14] X. Li, D.R. Stoll, P.W. Carr, Equation for peak capacity estimation in two-dimensional liquid chromatography, Anal. Chem. 81 (2008) 845–850.
[15] M. Camenzuli, P.J. Schoenmakers, A new measure of orthogonality for multidimensional chromatography, Anal. Chim. Acta 838 (2014) 93–101.
[16] M. Brokl, O. Hernández-Hernández, A.C. Soria, M.L. Sanz, M. L, Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides, J. Chromatogr. A 1218 (2011) 7697–7703.
[17] O. Hernández-Hernández, I. Calvillo, R. Lebrón-Aguilar, F.J. Moreno, M.L. Sanz, Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides, J. Chromatogr. A 1220 (2012) 57–67.
[18] J.H. Knox, P. Ross, Carbon-based packing materials for liquid chromatography - structure, performance, and retention mechanisms, Adv. Chromatogr. 37 (1997) 73–119.
[19] K. Koizumi, HPLC of carbohydrates on graphitized carbon column, in: Z. El Rassi (Ed.), Carbohydrates Analysis by Modern Chromatographic and Electrophoresis, Elsevier, The Netherlands, 2002, pp. 103–120.
[20] L. Montero, E. Ibañez, M. Russo, L. Rastrelli, A. Cifuentes, M. Herrero, Focusing and non-focusing modulation strategies for the improvement of on-line two-dimensional hydrophilic interaction chromatography x reversed phase profiling of complex food samples, Anal. Chim. Acta 985 (2017) 202–212.
[21] L. Montero, M. Herrero, M. Prodanov, E. Ibañez, A. Cifuentes, Characterization of grape seed procyanidins by comprehensive two-dimensional hydrophilic interaction x reversed phase liquid chromatography coupled to diode array detection and tandem mass spectrometry (HILICxRP LC – DAD – MS/MS), Anal. Bioanal. Chem. 405 (13) (2013) 4627–4638.
[22] A.F. Gargano, M. Duffin, P. Navarro, P.J. Schoenmakers, Reducing dilution and analysis time in online comprehensive two-dimensional liquid chromatography by active modulation, Anal. Chem. 88 (2016) 1785–1793.
[23] L. Coulier, J. Timmermans, R. Bas, R. Van Del Dool, I. Haaksman, B. Klarenbeek, T. Slahhek, W. Van Dogen, In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques, J. Agric. Food Chem. 57 (2009) 8488–8495.

5. DISCUSIÓN INTEGRADORA

La duda es el principio de la sabiduría.

Aristóteles

5. DISCUSIÓN INTEGRADORA

Como ha quedado patente en esta Tesis, el análisis de carbohidratos bioactivos no resulta una tarea trivial, al tratarse de mezclas muy complejas, con multitud de isómeros, con distintos grados de polimerización y distintas abundancias. Esto unido a la falta de patrones comerciales hace imprescindible el empleo de técnicas analíticas avanzadas con una alta resolución y capacidad de identificación. El avance que están experimentando las técnicas cromatográficas y espectrométricas actuales contribuye a una mejora del conocimiento, sin embargo, todavía queda mucho por hacer en este campo. En esta tesis se ha seguido una estrategia multianalítica, que ha permitido aprovechar las ventajas de cada una de las técnicas empleadas, desarrollando nuevos métodos alternativos a los existentes, que han supuesto una interesante contribución en este campo.

Como en cualquier proceso analítico, para la caracterización de carbohidratos es necesaria una etapa previa de preparación de muestra basada en este caso, principalmente, en el fraccionamiento de los compuestos de interés. La elección de la técnica de fraccionamiento más adecuada no es tarea fácil debido a la similitud de estructuras y a la necesidad de que los carbohidratos conserven su estructura química para no influir en su bioactividad. Además, si se trata de muestras reales esta etapa representa una dificultad añadida, debido al elevado contenido de algunos carbohidratos interferentes, como la lactosa en el caso de la leche o el calostro de cabra (> 98% de la fracción de carbohidratos) en comparación con los oligosacáridos presentes. Por tanto, esta etapa, generalmente es un cuello de botella en el análisis de carbohidratos. En esta tesis, para la eliminación de la lactosa de la leche de cabra se han empleados dos técnicas de fraccionamiento, una ampliamente establecida como es la cromatografía de exclusión molecular (SEC) y otra más novedosa basada en el empleo combinado de un tratamiento enzimático y microbiológico. La Tabla 5.1. muestra un resumen comparativo de las características de ambas técnicas.

Tabla 5.1. Características del fraccionamiento mediante cromatografía de exclusión molecular (SEC) y el tratamiento biotecnológico desarrollado en la Sección 3.1 basado en el uso combinado de β-galactosidasas de *K. lactis* y levaduras de *S. cerevisiae*.

	Disolventes	Volumen disolvente (L) / cantidad leche (g) desengrasada y desproteinizada	Eliminación lactosa (%)	Recuperación (%)	Coeficiente de variación (%)
SEC	Agua	1,25 / 1	99,9	Neutros DP3: 40-50 Ácidos DP3: 52-97 OS (≥DP4): ≈ 100	20-60
K. lactis + *S. cerevisiae*	Tampón fosfato pH 7	0,027 / 1	95	135 Aumento de COSg	1-20

La SEC es una técnica muy útil para la separación de carbohidratos en función de sus distintos grados de polimerización. Según los resultados obtenidos, prácticamente la totalidad de la lactosa pudo ser eliminada mediante SEC (99,9%), sin embargo, la recuperación de los distintos oligosacáridos varió en función de las estructuras de los mismos. Así, la recuperación de los trisacáridos neutros fue relativamente baja (40-50%), mientras que los ácidos y los OS de DP≥4 fue próxima al 100%, a excepción de la 3'SL que únicamente ser recuperó un 52%. Aunque la separación de los OS en SEC es relativa a su DP, la estructura de cada carbohidrato es fundamental. Posiblemente la 6'SL al tener una estructura más lineal se retiene menos que la 3'SL que eluye más próxima a la lactosa y por tanto su recuperación es menor. Mientras que los resultados relativos a la pureza de los OS son similares a los obtenidos previamente por otros autores para la purificación de GOS (Hernández et al., 2009), los rendimientos fueron inferiores en el caso de la leche de cabra (65-70% en COS *vs.* 81-92%, en GOS (Hernández et al., 2009)). Este comportamiento podría ser debido a las menores concentraciones de lactosa (≈20%) presentes en esta mezcla de prebióticos comparado con la leche de cabra. Por otra parte, el empleo de la SEC resulta tedioso (son necesarias aproximadamente 48 h para el proceso de equilibrado, separación cromatográfica y limpieza de la columna), la muestra se recupera muy diluida (el volumen de disolvente empleado para la elución es alto) y presenta problemas de reproducibilidad (la recuperación de carbohidratos para una muestra por triplicado presenta coeficientes de variación entre 20 y 60%). Por tanto, en la presente Memoria y considerando los problemas observados en SEC, los carbohidratos mayoritarios del calostro y la leche de cabra fueron analizados sin someter a la muestra a ningún proceso de fraccionamiento, mientras que los oligosacáridos de DP≥4, presentes en menor concentración, fueron analizados tras la eliminación de la lactosa mediante SEC.

Por otra parte, las técnicas de fraccionamiento no solo son útiles para el posterior análisis de la muestra, sino también para la obtención de carbohidratos con propiedades bioactivas a nivel preparativo. En este sentido, el tratamiento con enzimas y levaduras es una interesante alternativa, sin embargo, es imprescindible el estudio y optimización del proceso, ya que pueden producirse nuevos oligosacáridos por acción de enzimas transgalactosidasas y/o tener lugar la formación de productos secundarios no presentes inicialmente en la muestra por acción de las levaduras (Zhu y col., 2018). En la sección 3.1 de esta tesis se abordaron estos problemas y se pudieron obtener COS prácticamente libres de lactosa (eliminación del 95%) combinando β-galactosidasas y levaduras, minimizando la formación de nuevos oligosacáridos. Este método presentó una reproducibilidad adecuada (1-20%) y el procedimiento puede ser fácilmente adaptado para su implementación industrial.

Una vez obtenidos los oligosacáridos de interés resulta fundamental el desarrollo de métodos que permitan su análisis tanto cuali- como cuantitativo. En esta tesis, la caracterización de estos OS se ha desarrollado de manera exhaustiva gracias al uso de diversas técnicas cromatográficas complementarias. Para ello, se han usado técnicas avanzadas cromatográficas y espectrométricas como GC-Q MS, LC-Q MS, HPAEC-PAD, nano-LC-Chip-Q-ToF-MS, LC-IT MS y LC×LC-UV, que han resultado útiles para el análisis tanto de oligosacáridos de leche de cabra como de oligosacáridos prebióticos.

La GC-MS es una herramienta muy apropiada para el análisis de di- y trisacáridos debido a su sensibilidad y capacidad de resolución. Esta técnica, aunque de aplicación limitada en el caso de OS de alto grado de polimerización, permitió la caracterización detallada de los trisacáridos de la leche de cabra, a excepción de los derivados glicolil-neuramínicos, que, al presentar un mayor peso molecular, no pudieron ser analizados mediante esta técnica (Sección 3.2). La información estructural proporcionada por la GC-MS es superior a la LC-MS, presentando además un mayor poder de resolución, sin embargo, el análisis de carbohidratos ácidos y básicos es complejo y los procesos de derivatización deben ser cuidadosamente optimizados como ha quedado demostrado en esta Memoria.

Por otra parte, la cromatografía de líquidos permite el análisis de oligosacáridos de mayor DP. Como ya se ha comentado, son muchos los modos de operación de LC existentes, destacando la HPAEC, la GCC y la HILIC por su mayor poder de resolución entre carbohidratos isoméricos y con distinto grado de polimerización (Koizumi, 2002; Brokl et al., 2011). A pesar del alto poder de resolución proporcionado por la HPAEC, su difícil acoplamiento a MS, limita su aplicabilidad. Por ello, esta tesis se ha centrado principalmente en el empleo de GCC y HILIC. Así, se ha evaluado el uso de una columna de carbón grafitizado (PGC) y otra en base amida (BEH-Amide, trabajando en modo HILIC) para el análisis tanto de oligosacáridos de la leche de cabra (secciones 3.3 y 3.4) como para mezclas de di- y trisacáridos con distinta composición monomérica y enlaces glicosídicos (sección 4.1). El uso combinado de ambos modos de operación proporciona ventajas para el análisis de mezclas complejas de carbohidratos.

Además de las técnicas convencionales de LC –MS, en esta tesis se ha empleado la nano-LC-Chip-Q-TOF MS que es una técnica muy útil para el análisis de oligosacáridos, ya que permite la identificación de estos compuestos presentes en mezclas complejas introduciendo cantidades muy pequeñas de muestra. Gracias a las bases de datos existentes en la Universidad de Davis, durante una estancia en dicha Universidad, se consiguieron identificar 78 COS en calostro de cabra. Sin embargo, y dadas las fluctuaciones que presenta el voltaje del capilar del chip, la cuantificación de estos compuestos no es posible mediante esta técnica. Por tanto, la optimización de un método mediante HILIC-Q MS permitió la cuantificación de los principales OS presentes en esta muestra (Sección 3.3). Esta metodología se aplicó, posteriormente, con éxito para la evaluación del contenido de oligosacáridos de leches de cabra procedentes de distintas etapas de lactación, información no existente en la bibliografía hasta el momento (Sección 3.4).

En esta tesis se ha contemplado también el estudio de la fragmentación de di- y trisacáridos neutros mediante MS^n, empleando una trampa iónica como analizador. Este estudio permitió conocer los iones característicos de fragmentación de un gran número de disacáridos con distinta composición monomérica y enlaces glicosídicos, permitiendo establecer iones característicos para la identificación tentativa de estructuras más complejas como son los trisacáridos (sección 4.1).

Por otro lado, y a pesar de la capacidad de resolución de las técnicas monodimensionales y de los exitosos resultados obtenidos en las secciones anteriores, la caracterización de carbohidratos sería mucho más completa mediante el empleo de la cromatografía de líquidos bidimensional (LC×LC). Por tanto, en esta tesis nos planteamos desarrollar una nueva metodología mediante esta técnica para el análisis en primer lugar de mezclas de di- y trisacáridos y, posteriormente, aplicarlo a mezclas comerciales de carbohidratos prebióticos. Tras evaluar distintas fases estacionarias y basándonos en datos bibliográficos (Montero et al.), se observó que el empleo de columnas de fase inversa podía ser adecuado para su acoplamiento a HILIC o GCC. Sin embargo, los carbohidratos prácticamente no se retienen en columnas de RP, por lo que se consideró una etapa de derivatización previa con el reactivo ABEE, que aumentaría la retención de dichos compuestos en estas columnas. La derivatización de los carbohidratos además fue un requisito imprescindible para su detección mediante UV (detector acoplado al equipo de LC×LC), ya que estos compuestos carecen de grupos cromóforos. La optimización de las condiciones de separación demostró que la combinación HILIC×RP presentaba la mejor ortogonalidad. Sin embargo, para conseguir estos resultados, hubo que solventar problemas como las conexiones en la interfase o la incompatibilidad de las fases. El uso de dos columnas de C_{18} en la interfase para incrementar la retención antes de la segunda dimensión, junto con la introducción de un mecanismo adicional de modulación activa, resolvió los problemas de incompatibilidad de disolventes (sección 4.2). Cabe destacar que esta es la primera aplicación existente sobre el empleo de LC×LC para el análisis de carbohidratos. Sin duda alguna, esta es una nueva línea de investigación para poder continuar en el futuro, empleando detectores de MS y seleccionando las fases estacionarias y móviles adecuadas para conseguir la separación de mezclas complejas de carbohidratos sin necesidad de derivatización previa.

En base a lo arriba expuesto, los resultados incluidos en la presente Tesis Doctoral han contribuido de manera significativa al avance en el conocimiento sobre los procesos de fraccionamiento y análisis de carbohidratos bioactivos, objetivo encuadrado dentro del ámbito de Química Analítica.

6. CONCLUSIONES/ CONCLUSIONS

Solo hay un modo seguro de dar una vez en el clavo, y es dar ciento en la herradura.

Miguel de Unamuno

6. CONCLUSIONES

1. El uso combinado de β-galactosidasas de *Kluveromyces lactis* y levaduras de *Saccharomyces cerevisiae* ha permitido la eliminación selectiva de la lactosa presente en el calostro de cabra, consiguiendo preservar los oligosacáridos originalmente presentes y minimizando la formación de nuevos carbohidratos.

2. La nueva metodología desarrollada para la derivatización de oligosacáridos ácidos y neutros, utilizando *N*-trimetilsilil-*N*-metil trifluoroacetamida y trimetilclorosilano como agentes sililantes, ha resultado eficaz para su análisis por GC-MS. Dicho método ha sido aplicado con éxito para la caracterización de trisacáridos de leche de cabra.

3. El uso de nano-LC-Chip-Q-ToF MS ha permitido la identificación de hasta 78 oligosacáridos de calostro de cabra con unidades de hexosa, hexosamina, fucosa, ácido *N*-acetilneuramínico y/o ácido *N*-glicolilneuramínico, siendo la primera vez que se lleva a cabo un estudio de caracterización tan completo de los carbohidratos de este producto.

4. El nuevo método desarrollada mediante HILIC-Q MS ha contribuido a la separación y la cuantificación de 29 oligosacáridos del calostro de cabra, tanto neutros como ácidos, siendo los carbohidratos predominantes las galactosil-lactosas seguidas por los sialil- y fucosil-oligosacáridos, respectivamente.

5. La metodología optimizada mediante nano-LC-Chip-Q-ToF MS y HILIC-Q MS ha permitido, por primera vez, el estudio exhaustivo de la evolución de los oligosacáridos de leche de cabra, tanto neutros como ácidos, durante diferentes etapas de lactación.

6. La combinación de datos del comportamiento cromatográfico (basado en el empleo de columnas HILIC y PGC) y espectrométrico (MS^n) de patrones de disacáridos con diferentes enlaces glicosídicos y unidades monoméricas ha permitido establecer nuevos criterios para la caracterización tentativa de estructuras más complejas, aplicándose al análisis de trisacáridos.

7. La cromatografía de líquidos bidimensional completa (LC×LC) se ha empleado, por primera vez, para el análisis de carbohidratos, resultando de utilidad para la separación de mezclas complejas de patrones de di- y trisacáridos previamente derivatizados con ácido paraaminobenzoico.

8. La metodología de HILIC×RP desarrollada ha permitido la separación de mezclas complejas de oligosacáridos prebióticos comerciales, cuya resolución no era posible en una dimensión, abriendo una nueva vía de investigación para futuras aplicaciones en este campo.

De manera genérica, se puede afirmar que los trabajos desarrollados en el marco de esta Tesis Doctoral han contribuido al avance del conocimiento relacionado con el desarrollo de metodologías para el análisis de mezclas complejas de carbohidratos bioactivos, proponiendo soluciones a diversas problemáticas existentes, tanto en la etapa de preparación de muestra como en el análisis mediante el uso de técnicas cromatográficas y espectrométricas.

CONCLUSIONS

1. The combined use of β-galactosidases from *Kluveromyces lactis* and yeast from *Saccharomyces cerevisiae* has proven to be efficient in removing lactose from goat colostrum, preserving its endogenous oligosaccharides and minimising the synthesis of new oligosaccharides.

2. The new methodology developed for the derivatization of acidic and neutral oligosaccharides, using *N*-trimethylsilyl-*N*-methyl trifluoroacetamide and trimethylchlorosilane as silylating agents, has demonstrated to be efficient for their analysis by GC-MS. This method has been successfully applied to the characterization of goat milk trisaccharides.

3. The use of nano-LC-Chip-Q-TOF MS has allowed the identification of up to 78 oligosaccharides from goat colostrum consisting of hexose, hexosamine, fucose, *N*-acetylneuraminic acid and/or *N*-Glycolylneuraminic acid. This is the first time that such a comprehensive study has been carried out about the oligosaccharide composition of goat colostrum.

4. The new methodology developed by HILIC-Q MS has allowed the separation and quantification of 29 oligosaccharides from goat colostrum, both acidic and neutral, galactosyl-lactoses being the predominant carbohydrates followed by sialyl- and fucosyl-oligosaccharides, respectively.

5. The optimized method by nano-LC-Chip-Q-TOF MS and HILIC-Q MS has permitted, for the first time, the comprehensive study of the evolution of goat milk oligosaccharides, both acidic and neutral, during different lactation stages.

6. The data combination from chromatographic (based on the use of HILIC and PGC columns) and spectrometric (MS^n) behaviours of disaccharide standards having different glycosidic linkages and monomeric units has provided new insights into the tentative characterization of more complex carbohydrate structures as those of trisaccharides .

7. Comprehensive two-dimensional liquid chromatography (LC×LC) has been applied, for the first time, to the analysis of carbohydrates, being successful for the separation of complex mixtures of di- and trisaccharide standards previously derivatized with para-aminobenzoic acid.

8. The developed methodology based on the use of HILIC×RP has allowed the separation of complex mixtures of commercial prebiotic oligosaccharides, which were unable to be separated in one dimension, open the door to further applications in the field of carbohydrates analysis.

Overall, it may be concluded that the developed work presented in this PhD thesis has generated a progress in the state-of-art of methodology development for the analysis of complex mixtures of bioactive carbohydrates, providing solutions to current challenges related to sample preparation and analysis by using chromatographic and spectrometric techniques.

7. ANEXOS

El científico no es aquella persona que da las respuestas correctas, sino aquél quien hace las preguntas correctas.

Claude Lévi-Strauss

Anexo 1: Correspondiente a la sección 1

Análisis de carbohidratos bioactivos mediante cromatografía líquida de interacción hidrofílica (HILIC)

M.J. García-Sarrió, S. Rodríguez-Sánchez, A. Martín-Ortiz

Cromatografía y Técnicas Afines, 2014, 35(2), 47-58.

ARTÍCULOS

Análisis de carbohidratos bioactivos mediante cromatografía líquida de interacción hidrofílica (HILIC)

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1. INTRODUCCIÓN

Los carbohidratos son la principal fuente de energía de los seres vivos y son, además, uno de los constituyentes más importantes de los alimentos: contribuyen a la mejora de sus propiedades organolépticas, permiten la modificación de texturas, son empleados como marcadores de calidad, etc. Sin embargo, además de estas funciones, dependiendo de su estructura química, algunos carbohidratos poseen efectos beneficiosos para la salud humana, por lo que son susceptibles de ser incluidos como ingredientes en la elaboración de muchos alimentos. Entre estos carbohidratos destacan los iminoazúcares y los oligosacáridos prebióticos, objeto de estudio en este artículo.

A la hora de considerar un carbohidrato o una mezcla de carbohidratos como bioactivos es necesario determinar su composición química y su pureza (FAO, 2007). Por lo tanto, es de gran interés el desarrollo de métodos analíticos adecuados que permitan caracterizar los carbohidratos bioactivos para establecer relaciones estructura-función.

Los carbohidratos bioactivos, tanto los presentes en la naturaleza como los obtenidos mediante procesos de síntesis, generalmente forman parte de mezclas complejas de distintos grado de polimerización (DP), composición monomérica, enlaces glicosídicos, ramificaciones y formas anoméricas. Además de su gran variabilidad estructural, los carbohidratos son compuestos muy polares, de baja estabilidad térmica y gran reactividad química, que no poseen grupos fluorescentes ni cromóforos y no son volátiles (Ikegami y col., 2008; Brokl, 2011). Por tanto, su caracterización supone un reto desde el punto de vista analítico, siendo necesaria la selección de técnicas y optimización de condiciones experimentales que proporcionen una mayor resolución e información estructural. Además, estos carbohidratos bioactivos se encuentran frecuentemente presentes en baja concentración en fuentes naturales, por lo que su determinación requiere el uso de técnicas analíticas sensibles.

La cromatografía de líquidos (LC) es una de las técnicas más usadas para la caracterización de carbohidratos debido a su gran versatilidad y múltiples modos de operación. Entre los modos más empleados se encuentra la cromatografía líquida en fase inversa (RPLC), la cromatografía de alta eficacia de intercambio aniónico (HPAEC) y la cromatografía líquida de interacción hidrofílica (HILIC) (Antonio y col., 2008), destacando en los últimos años el número de aplicaciones de esta última por su elevado poder de resolución para compuestos polares y facilidad de acoplamiento a espectrometría de masas, como se discutirá en la sección 2.

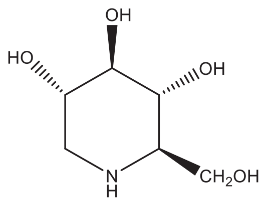
1.1. Iminoazúcares

¿Qué son?
Los iminoazúcares, también llamados azazúcares y polihidroxialcaloides, son carbohidratos bioactivos de bajo peso molecular con estructura similar a la de los monosacáridos, en los que el átomo de oxígeno del anillo se ha reemplazado por un átomo de nitrógeno.

¿Qué tipos hay?
Dependiendo de su estructura química se clasifican en cinco grupos: piperidinas (ej. 1-desoxinojirimicina (DNJ) y α-homonojirimicina (α-HNJ)), pirrolidinas (ej. 2,5-didesoxi-2,5-imino-D-manitol (DMDP)), indolicidinas (ej. swainsonina), pirrolicidinas (ej. australina) y nortropanos (ej. calisteginas). En la **Figura 1** se muestran las estructuras de los iminoazúcares mencionados.

En la naturaleza, los iminoazúcares se encuentran presentes en numerosas familias de plantas (Moraceae, Leguminosae, Hyacinthaceae, Araceae, etc.) (Asano y col., 2005) y en diversos géneros de microorganismos (*Streptomyces*, *Bacillus*, etc.) (Onose y col., 2013). Dentro de las fuentes de origen vegetal cabe destacar la morera (*Morus* sp.) y el trigo sarraceno o alforfón (*Fagopyrum esculentum*) por su mayor disponibilidad e interés alimentario.

¿Qué propiedades tienen?
Los iminoazúcares son conocidos inhibidores competitivos de glicosidasas. Esta actividad se basa en su propiedad de mimetizar la estructura de los azúcares sencillos, lo que permite que puedan ocupar el lugar de éstos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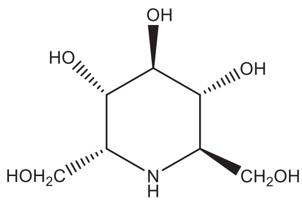
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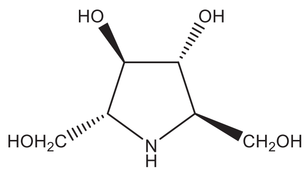
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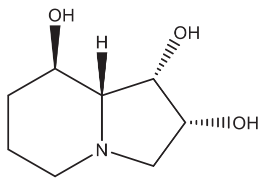
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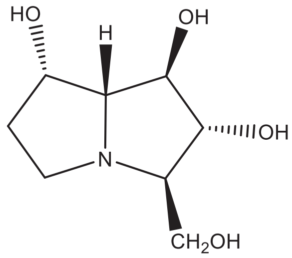
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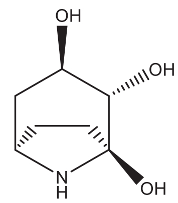
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Figura 1. Estructuras químicas de DNJ (1), α-HNJ (2), DMDP (3), swainsonina (4), australina (5) y calistegina A_3 (6).

en reacciones catalizadas por las enzimas que digieren dichos azúcares (Watson, y col., 2001).

Esta actividad inhibitoria les confiere gran utilidad como candidatos para el tratamiento de numerosas enfermedades relacionadas con la absorción de carbohidratos, tales como obesidad y diabetes, de gran incidencia en países desarrolados (Gómez y col., 2012). También los iminoazúcares se han propuesto para el tratamiento de distintos tipos de cáncer, infecciones virales, etc.

Entre los iminoazúcares naturales con mayor actividad antiglicosidasa se encuentran la DNJ, la fagomina (1,5-imino-1,2,5-trideoxi-D-arabino-hexitol), la α-HNJ y el DMDP (Evans y col., 1985).

¿Por qué es difícil su análisis?
La principal dificultad de su análisis es debida al elevado número de isómeros de estos carbohidratos, que generalmente se encuentran en relativamente baja concentración y formando parte de muestras complejas, al igual que la ausencia de patrones comerciales con los que poder compararlos.

1.2. Prebióticos

¿Qué son?
Son ingredientes que producen una estimulación selectiva del crecimiento y/o actividad(es) de uno o de un limitado número de géneros/especies de microorganismos en la microbiota intestinal, confiriendo beneficios para la salud del hospedador (Roberfroid y col., 2010). Para conseguir este crecimiento selectivo y que, en consecuencia, un compuesto sea considerado como prebiótico, debe cumplir los siguientes requisitos (Steed y col., 2009):

i) Resistir los bajos valores de pH gástricos, la acción de enzimas digestivas y de sales biliares.

ii) No ser hidrolizado o absorbido en el tracto gastrointestinal en su totalidad y llegar al intestino grueso, sin variaciones en su estructura molecular.

iii) Ser fermentado selectivamente por bacterias beneficiosas de la microbiota intestinal.

iv) Ser capaz de inducir efectos beneficiosos para la salud.

¿Qué tipos hay?

Los prebióticos son, principalmente, oligosacáridos de diversos DP, aunque también se pueden considerar algunos disacáridos (ej. lactulosa) o polisacáridos. En Europa existen actualmente cuatro productos con reconocidas propiedades prebióticas establecidos en el mercado (Gosling y col., 2010): galactooligosacáridos (GOS), fructooligosacáridos (FOS), inulina y lactulosa. La **Figura 2** muestra algunos ejemplos de estos carbohidratos.

¿Qué propiedades tienen?

Entre las propiedades beneficiosas que pueden ejercer los prebióticos destacan la inhibición del crecimiento de bacterias exógenas y perjudiciales, el favorecimiento de la absorción de minerales, la estimulación del sistema inmune y la producción de vitaminas (Roberfroid y col., 2010)

¿Por qué es difícil su análisis?

El análisis de carbohidratos prebióticos supone un gran reto para la investigación. La similitud estructural de los carbohidratos prebióticos, junto con su frecuente presencia en forma de mezclas complejas de distinto DP y en un amplio intervalo de concentraciones, son algunas de las dificultades que entraña el análisis de estos carbohidratos desde el punto de vista cromatográfico. Por tanto, a veces es necesario llevar a cabo un fraccionamiento de la muestra previo al análisis con el fin de obtener fracciones de un DP determinado y simplificar así su caracterización. El hecho de que prácticamente no existan patrones comercialmente disponibles dificulta aún más su identificación.

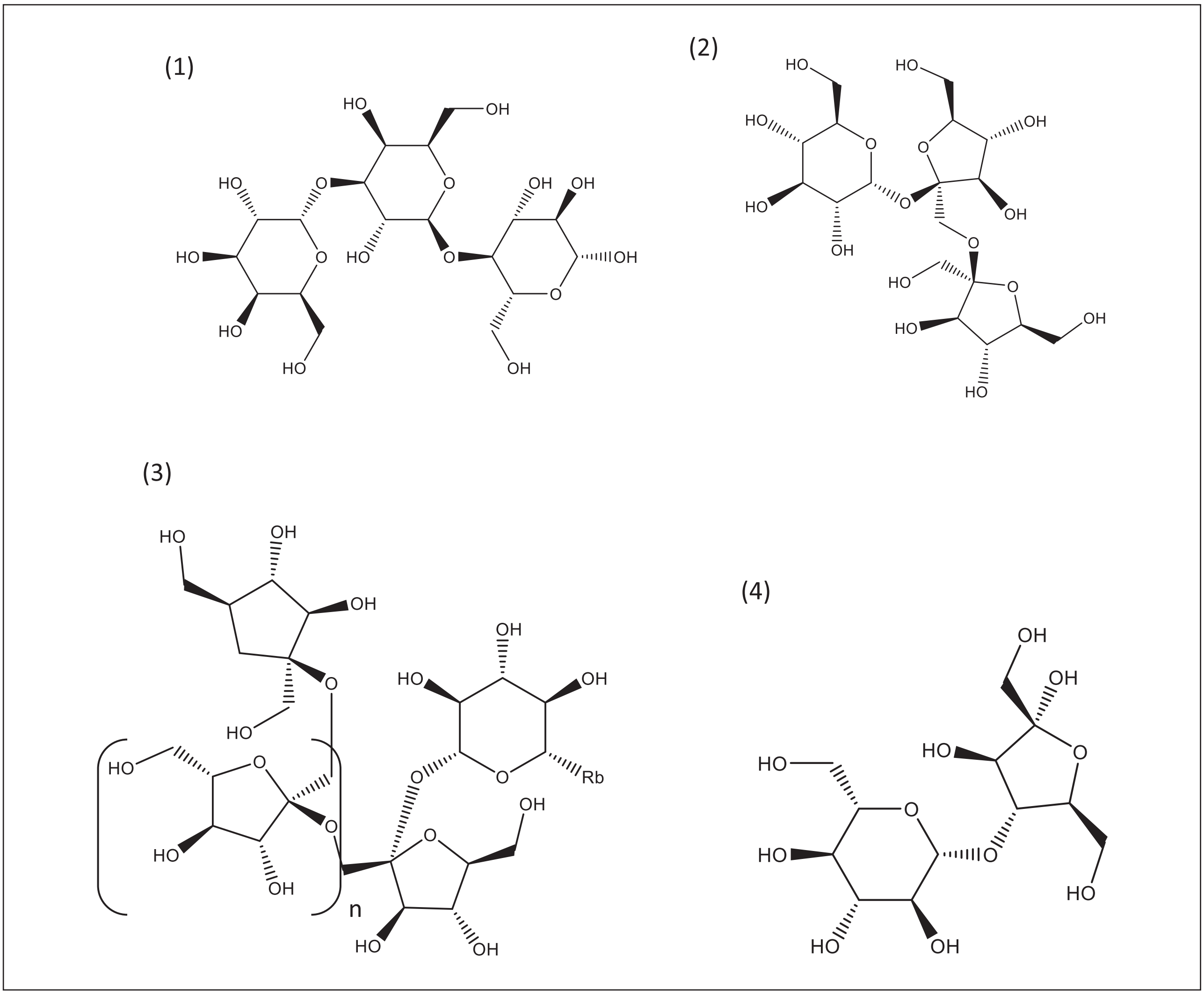


Figura 2. Ejemplo de algunos carbohidratos reconocidos como prebioticos: (1) 3'-galactosil-lactosa (GOS), (2) 1-kestosa (FOS), (3) inulina y (4) lactulosa.

2. CROMATOGRAFÍA LÍQUIDA DE INTERACCIÓN HIDROFÍLICA (HILIC)

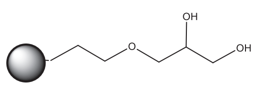
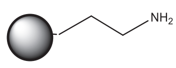
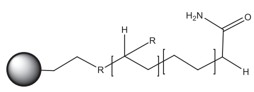
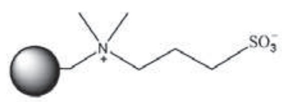
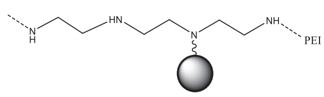
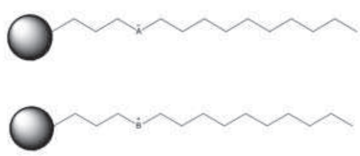
La HILIC es un modo de HPLC, inicialmente desarrollado para la separación de carbohidratos, y que proporciona en la actualidad excelentes resultados en la separación de otras moléculas como aminoácidos, vitaminas, toxinas, etc. HILIC se presenta frecuentemente como una variante de la cromatografía de fase normal (NPLC) puesto que al igual que ésta, utiliza una fase estacionaria polar y la retención de los analitos aumenta con el incremento de su hidrofilia, aunque su mecanismo es mucho más complejo (Greco y Letzel, 2013).

Resulta de gran utilidad para analizar compuestos que, dada su elevada polaridad, se retienen poco en RPLC y eluyen cerca del frente del disolvente, o no dan lugar a una retención efectiva en cromatografía de intercambio iónico. Los analitos polares siempre muestran una buena solubilidad en la fase móvil acuosa utilizada en HILIC, lo que contribuye a solventar los frecuentes problemas relacionados con la baja solubilidad de estos compuestos en las fases de NPLC.

2.1. Fases estacionarias

Aunque cualquier fase estacionaria que pueda retener moléculas de agua, puede ser usada en modo HILIC (Greco y Letzel, 2013), la fase estacionaria típica consiste en la clásica gel de sílice modificada con distintos grupos funcionales polares (amino, diol, amida, etc.; ver **Tabla 1**). Las reconocidas ventajas de la utilización de fases estacionarias hidrofílicas para la separación de compuestos polares, han potenciado en los últimos años el desarrollo de nuevas generaciones de fases estacionarias HILIC, la mayoría de las cuales presentan mecanismos de interacción mixtos basados generalmente en la inclusión de grupos intercambiadores iónicos o grupos zwitteriónicos (Buszewski y Noga, 2011; ver **Tabla 1**). En general, las fases estacionarias HILIC se pueden agrupar en neutras, polares o iónicas.

Tabla 1. Ejemplos de fases estacionarias de sílice con distintos grupos funcionales empleadas en HILIC.

Fases estacionarias	Estructura de la fase estacionaria
diol	
amino	
amida	
zwitteriónicas (tipo sulfobetaina)	
intercambio iónico*	
mixtas RP/intercambio iónico	

*PEI: Polietilenimina

2.2. Fases móviles

La fase móvil en HILIC está constituida por una mezcla hidro-orgánica, cuyo porcentaje en agua aumenta con el tiempo de análisis. Una elevada proporción inicial de disolvente orgánico en la mezcla produce que el agua sea fuertemente adsorbida en la superficie de la fase estacionaria hidrofílica. Cuando la cantidad de agua constituye alrededor de un 5% de la fase móvil, la capa de agua adsorbida es lo suficientemente delgada como para crear un sistema de reparto líquido-líquido con la fase orgánica (Greco y Letzel, 2013).

Como disolvente orgánico generalmente se emplea acetonitrilo, aunque se puede utilizar cualquier disolvente aprótico miscible con agua, como tetrahidrofurano o dioxano. Los alcoholes (como metanol o etanol) se pueden también emplear aunque es necesaria una mayor concentración para obtener el mismo grado de retención relativo de los analitos (Hemström y col., 2006). En general, la fuerza de elución para los distintos disolventes utilizados en HILIC aumenta en el orden siguiente: acetonitrilo < tetrahidrofurano < isopropanol y propanol < etanol < metanol < agua (Greco y Letzel, 2013). Además, suelen emplearse aditivos iónicos como el acetato amónico o formiato amónico para controlar el pH y la fuerza iónica de la fase móvil, lo que contribuye a pequeños cambios en la retención (Buszewsky y Noga, 2011).

Por otra parte, es necesario señalar también que el empleo en HILIC de fases móviles del todo compatibles con la Espectrometría de Masas (MS) ha contribuido a la creciente popularidad del acoplamiento directo de ambas técnicas (HILIC-MS).

2.3. Mecanismo de retención

El mecanismo de retención en HILIC es muy complejo y todavía genera controversia entre la comunidad científica. Se ha demostrado que el patrón de elución en HILIC es similar al de la fase normal, por lo que se podría presuponer que el mecanismo debería tener también alguna similitud (Buszewsky y Noga, 2011).

Una de las teorías actualmente aceptadas sostiene que la retención en HILIC es principalmente causada por un proceso de reparto, si bien no se conoce en profundidad su fundamento teórico. Según dicha teoría, la diferente distribución de las moléculas del analito entre el acetronitrilo de la fase móvil y la capa de agua adsorbida sobre la fase estacionaria hidrofílica darían lugar a la separación (Buszewsky y Noga, 2011) (**Figura 3**). Cuanto más hidrofílico sea el analito, más se desplazará el equilibrio hacia la capa de agua inmovilizada en la fase estacionaria, y más se retendrá el analito. La separación, por tanto, no sólo se basa en las polaridades de los compuestos sino también en su grado de solvatación.

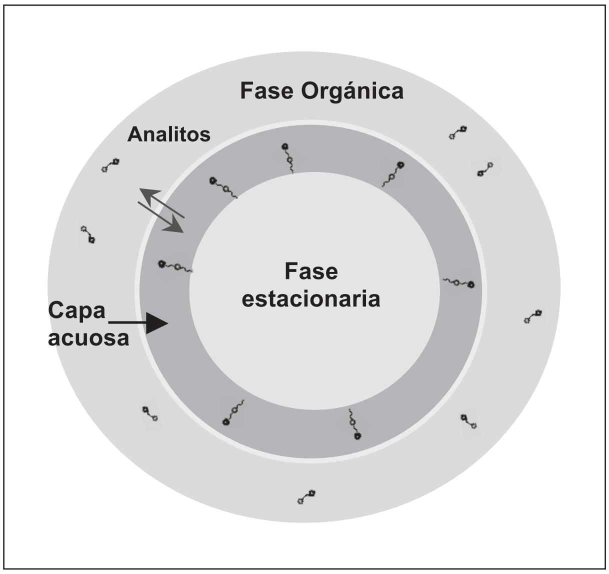


Figura 3. Esquema del mecanismo de retención en HILIC.

Pero el mecanismo en HILIC es más que un simple reparto, e incluye también interacciones tipo puente de hidrógeno entre especies neutras y polares, así como interacciones electrostáticas débiles con la fase estacionaria (Alpert, 1990; Yoshida, 2004). Estas interacciones electrostáticas pueden deberse a cargas residuales de grupos silanoles disociados, que han quedado bloqueados en la fase estacionaria debido a efectos estéricos y no pueden ser eliminados, o a grupos que han sido intencionadamente incorporados a la fase estacionaria (Buszewsky y Noga, 2011). En este último caso, son varios los mecanismos que deben considerarse para explicar la retención, siendo únicamente posible el determinar el mecanismo predominante con la experimentación.

3. ANÁLISIS DE IMINOAZÚCARES

En los últimos años, el desarrollo de nuevos métodos para la separación e identificación de iminoazúcares con potencial uso terapéutico ha cobrado un especial interés (Watson y col., 2001). Como se ha comentado en la Introducción, considerando la variada bioactividad de los distintos iminoazúcares, resulta necesario determinar sus estructuras de forma previa a su uso como ingredientes bioactivos. Existen diversos estudios que analizan iminoazúcares mediante cromatografía de gases (GC), sin embargo, la necesidad de convertir estos compuestos en derivados más volátiles y estables previo a su análisis, hace que la LC se presente como una alternativa de gran interés. Aunque se han utilizado distintos modos de operación como HPAEC (Yoshihashi y col., 2010), RPLC (Xie, 2008), NPLC (Ritchie y col., 2010), etc, en los últimos años la HILIC ha surgido como una herramienta eficaz para el análisis de estos carbohidratos bioactivos.

La mayoría de los estudios más recientes se han basado en la determinación de DNJ, un iminoazúcar que como ya se ha comentado, ha sido investigado por su potencial uso contra la diabetes, al ser un inhibidor eficaz de la actividad α-glucosidasa. Kimura y col. publicaron en 2004 un estudio en el que se determinaba este iminoazúcar de forma simple y rápida en hojas de morera, utilizando una columna de sílice funcionalizada con grupos amida (de dimensiones 250 mm longitud $\times$ 4,6 mm d.i.) y una mezcla de acetonitrilo:agua (81:19, v/v) conteniendo 6,5 mM de acetato amónico a un pH de 5,5 como fase móvil. La elución se llevó a cabo en modo isocrático a una temperatura de 40 ^{o}C. El flujo de la fase móvil (1 mL min^{-1}) era dividido después de la columna: un flujo de 0,95 mL min^{-1} era enviado a un detector de dispersión de luz (ELSD) y uno de 0,05 mL min^{-1} era enviado a un detector de espectrometría de masas con analizador de tiempo de vuelo (TOF MS), empleándose este último detector para confirmar la identidad del DNJ. Previo a la selección de la columna HILIC, se probaron columnas clásicas de LC de fase normal e inversa en diferentes condiciones, pero no se obtuvieron retenciones adecuadas para dicho iminoazúcar.

En 2007, Nuengchamnong y col. utilizaron la misma columna que Kimura y col. (2004) para la cuantificación mediante cromatografía liquida acoplada a espectrometría de masas tándem (LC-MS/MS) de DNJ en hojas de morera. Sin embargo, estos autores emplearon un medio ácido usando como fase móvil 0,1% de ácido fórmico (eluyente A) y acetonitrilo (eluyente B) y utilizando un programa inicialmente isocrático seguido de una rampa que, curiosamente y a diferencia de Kimura, empezó en proporciones 80:20 (v/v, A:B) hasta 10:90 (v/v, A:B). El flujo utilizado fue de 0,6 mL min^{-1}. El método propuesto supuso una reducción considerable del tiempo de análisis (el tiempo de retención del DNJ varió de 24,5 a 8,1 min), proporcionando además una alta sensibilidad y selectividad en la determinación de este iminoazúcar. Además, los resultados mostraron que existían diferencias en los niveles de DNJ con la etapa de crecimiento de las hojas, observándose mayores niveles de DNJ en hojas jóvenes.

En 2010, Nakagawa y col. propusieron un método por HILIC-MS/MS para la determinación de DNJ, galactosil-DNJ (GAL-DNJ) y fagomina en hojas de morera y productos de sericicultura. En este caso también se utilizó la misma fase amida que en los casos anteriores y una fase móvil con una mayor concentración de ácido fórmico que la utilizada por Nuengchamnong tres años antes (0,1% de ácido fórmico, en ambas fases). El gradiente empleado fue también significativamente diferente, empezando con una relación de acetonitrilo/agua 80:20 (v/v), para alcanzar en 2 minutos una proporción 40:60 (v/v), que se mantenía durante 2,5 min antes de restablecer las condiciones iniciales para el equilibrado de la columna. El empleo de un detector de masas requirió un flujo bajo (0,2 mL min^{-1}) y la temperatura se mantuvo en 40 ^{o}C. En estas condiciones, se consiguió la resolución de DNJ, GAL-DNJ y fagomina en un tiempo análisis inferior a 6 minutos (**Figura 4**).

El método desarrollado, además de selectivo, fue lo suficientemente sensible para el análisis de iminoazúcares en las muestras de interés. Así, los resultados obtenidos probaron que, en contraste con las hojas de morera, los gusanos de seda presentaban una concentración alta de DNJ, moderada de fagomina y baja de GAL-DNJ, lo que sugirió que cuando los gusanos se alimentaban de las hojas de morera, el DNJ y la fagomina eran absorbidos sin sufrir modificaciones, pero el GAL-DNJ era metabolizado en forma de DNJ. Este último iminoazúcar no sólo presenta un efecto inhibidor de la α-glucosidasa, sino que también resulta ser inhibidor del crecimiento de insectos y muestra propiedades insecticidas, de ahí su acumulación en los gusanos como sistema de defensa. En este trabajo además se observaron diferentes concentraciones de iminoazúcares en las hojas de las distintas variedades de morera consideradas.

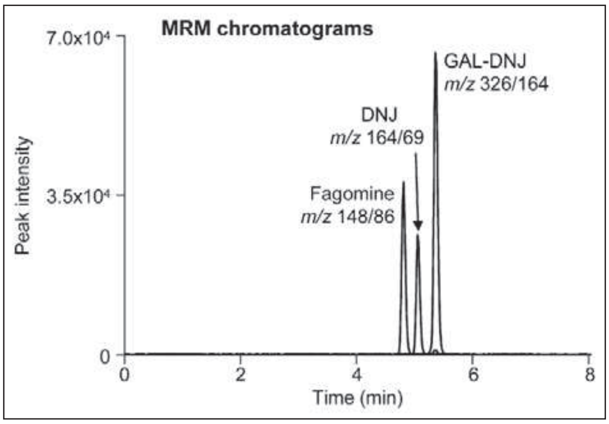


Figura 4. Separación de Fagomina, GAL-DNJ y DNJ mediante HILIC-MS/MS. Reproducida con permiso de Elsevier (Nakagawa y col., 2010).

En 2009, Guitton y col. desarrollaron un método mediante HILIC-MS para el análisis cuantitativo de miglustat en plasma y fluido cerebroespinal humano. Miglustat es el nombre comercial con el que se introdujo en el mercado la *N*-butil-desoxinojirimicina, un iminoazúcar inhibidor de la glucosiltransferasa que cataliza el primer paso de la síntesis de glicoesfingolípidos (GSLs). Varias mutaciones genéticas producen una acumulación patógena de los GSLs en los lisosomas, que conducen a los desórdenes encontrados en varias enfermedades como las de Tay-Sachs, Gaucher or Niemann-Pick (Platt y col., 1998).

En este caso, la separación de analitos se llevó a cabo con una columna HILIC de sílice (150 × 2,1 mm, 3 μm), manteniendo la temperatura a 25 ºC. El eluyente utilizado en la separación en modo isocrático fue una mezcla de acetonitrilo/agua/acetato amónico 100 mM (pH 5) en proporciones 75/10/15 (v/v/v), con un flujo de 0,23 mL min^{-1}. Las condiciones seleccionadas dieron lugar a la elución del miglustat como un pico estrecho y simétrico sin interferencias de las correspondientes matrices biológicas (plasma o fluido cerebroespinal). Este método proporcionó ventajas en cuanto a selectividad y tiempos de retención (t_R miglustat = 3,7 min) en comparación con los métodos previos descritos en la bibliografía y basados en otros modos de LC (Mellor y col., 2000). Además, la buena exactitud y precisión obtenidas por este método permitieron su validación para el seguimiento en la administración de miglustat en pacientes con enfermedades relacionadas con los GSLs.

Recientemente, Rodríguez-Sánchez y col. (2014) evaluaron el uso de diferentes fases estacionarias hidrofílicas para el análisis simultáneo de patrones de iminoazúcares (DNJ, α-HNJ, 1-desoximannojirimicina (DMJ), miglitol ((2R,3R,4R,5S)-1-(2-hydroxyethyl)-2-(hydroxymethyl) piperidine-3,4,5-triol), *N*-metil-DNJ y DMDP) y otros carbohidratos de bajo peso molecular (LMWC) (fructosa, glucosa, *myo*-inositol, sacarosa y galactinol). Las columnas evaluadas fueron las siguientes: i) una fase estacionaria de polihidroxietil-aspartamida (100 x 2,1 mm); ii) una fase zwitteriónica tipo sulfoalquilbetaina (150 x 2,1 mm); y iii) una fase tipo amida (150 x 4,6 mm). Para cada una de las columnas se probaron diferentes flujos y gradientes de una fase móvil binaria consistente en acetonitrilo:agua con diferentes modificadores orgánicos (0,1% de ácido acético y 0,1% de hidróxido amónico). El análisis estadístico de los resultados, considerando como variables la resolución, el tiempo de retención y la simetría de los picos, mostró como fase estacionaria óptima la columna tipo amida, empleando 0,1% hidróxido amónico como aditivo de la fase móvil, a diferencia de los trabajos previamente descritos donde se utilizaba ácido fórmico o diversas sales. Las condiciones óptimas se aplicaron al análisis de iminoazúcares y otros LMWC en extractos de hojas de morera, de semillas de trigo y de bulbos de jacinto.

Para complementar estos resultados, y empleando la columna previamente seleccionada, los mismos autores evaluaron el efecto de distintos modificadores orgánicos de la fase móvil (metanol y acetonitrilo), aditivos (ácido fórmico, ácido acético, acetato amónico e hidróxido amónico) y temperaturas de la columna (25, 45, 55 y 65 ºC) para el análisis de iminoazúcares y otros compuestos de bajo peso molecular en extractos de *Aglaonema* sp. Las condiciones óptimas resultaron ser acetonitrilo:agua con 0,1% hidróxido amónico como fase móvil y 55 ºC. El acoplamiento de HILIC a la espectrometría de masas (QTOF MS) en este trabajo permitió la caracterización de diversos iminoazúcares, algunos de ellos detectados por primera vez, en extractos procedentes de distintas especies de aglaonema (García-Sarrió y col., 2014).

4. ANÁLISIS DE PREBIÓTICOS

Debido a las limitaciones de la GC para el análisis de oligosacáridos de alto DP, la LC suele ser la técnica de elección para el análisis de carbohidratos prebióticos. Algunos trabajos, como el de Brokl y col. (2011), evalúan distintos modos de operación de LC para el análisis de estos compuestos: RPLC, HPAEC, cromatografía de carbón grafitizado (GCC) y HILIC. Los mejores resultados se obtuvieron empleando GCC y HILIC. Mientras que la GCC permitió una separación adecuada para oligosacáridos del mismo DP y distinto enlace glicosídico, la HILIC proporcionó los mejores resultados para el análisis de mezclas de oligosacáridos con distintos DPs. En general, en HILIC los oligosacáridos eluyen en función de su DP y su retención es inversamente proporcional al aumento de agua en la fase móvil (Alpert, 1990).

En la **Tabla 2** se muestran algunos ejemplos de aplicaciones recientes de la HILIC para el análisis de diferentes tipos de carbohidratos prebióticos.

Tabla 2. Ejemplos del análisis de distintos tipos de carbohidratos prebióticos mediante HILIC.

PREBIÓTICO	COLUMNA	CONDICIONES	REFERENCIA
GOS	Amida (150 x 4,6 mm; 3,5 µm)	0,4 mL min^{-1}; 35 ºC ACN/H_2O + 0,1% NH_4OH (80:20, v/v) → (50:50, v/v) en 31 min	Hernández-Hernández y col., 2011
	Polimérica (300 x 20 mm; 9 µm) Semipreparativa	10 mL min^{-1}; 21 ºC DP2: ACN/H_2O (80:20, v/v) DP3: ACN/H_2O (76,5:23,5 v/v)	Coulier y col., 2009
	Zwitteriónica tipo sulfobetaína (150 x 2,1 mm; 5 µm)	0,1 mL min^{-1} MeOH+ 5% NH_4OAc/H_2O (95:5, v/v) → (50:50, v/v) en 40 min	Sinclair y col., 2010
	Maltosa "click" semipreparativa (100 x 10 mm)	3 mL min^{-1}; 30 ºC ACN/H_2O (70:30, v/v) 10 min → (40:60, v/v) 10 min	Fu y col., 2010
FOS	Maltosa "click" analítica (150 x 4,6 mm)	1 mL min^{-1}; 30 ºC H_2O/ACN (30:70, v/v) → (50:50, v/v) en 60 min	Fu y col., 2010
INULINA	β-ciclodextrina (250 x 4,6 mm; 5 µm)	0,4 mL min^{-1}; 26 ºC ACN/H_2O (70:30, v/v)	Wang y col., 2010
LACTULOSA	ZIC®-HILIC (250 × 2,1 mm; 5 µm)	0,3 mL min^{-1} ACN/NH_4OAc 5 mM (75:25, v/v) → (40:60, v/v) en 10 min	Kubica y col., 2012

La mayor parte de los estudios se han centrado en el desarrollo de métodos de análisis de GOS, mezclas complejas de oligosacáridos basados en unidades de galactosil-galactosa y galactosil-glucosa, producidos mediante transgalactosilación de la lactosa, usando como catalizador β-galactosidasas de diferentes orígenes biológicos.

Sinclair y col. (2009) consiguieron una buena separación de estos oligosacáridos empleando una columna zwitteriónica tipo sulfobetaína (150 × 2,1 mm d.i., 5 µm), una fase móvil constituida por metanol con 5 mM de acetato amónico (fase A) y agua (fase B) y un gradiente que varió de 95:5 (v/v, A:B) hasta 50:50 (v/v, A:B) en 40 min.

Recientemente, Hernández-Hernández y col. (2011) evaluaron el empleo de tres fases estacionarias de sílice funcionalizadas con diferentes grupos (zwitteriónica de tipo sulfobetaína, polihidroxietilaspartamida y amida) con el fin de obtener tiempos de retención cortos, picos con buenas simetrías y resoluciones mayores de 1 en el análisis de GOS. Para la optimización del método se emplearon mezclas de patrones comerciales con distintos DP y enlaces glicosídicos. En las tres columnas se observaron diferencias en la forma de los picos, en su retención y en su selectividad debidas a la distinta naturaleza de las fases estacionarias. Las mejores condiciones se obtuvieron empleando la columna tipo amida y usando como fase móvil acetonitrilo:agua con 0,1% de hidróxido amónico. La **Figura 5** muestra los perfiles obtenidos para una mezcla de GOS comercial (DP2-DP5) analizada empleando estas condiciones. Aunque las separaciones obtenidas fueron adecuadas, la resolución completa de todos los oligosacáridos presentes en esta mezcla compleja no fue posible. Gracias al empleo de un detector de masas con analizador de trampa de iones (IT), que permitió la obtención de espectros de masas en tándem (MS^n), se consiguió identificar tentativamente los enlaces glicosídicos de estos oligosacáridos, empleando las reglas de fragmentación deducidas del análisis previo de distintos patrones. Los resultados de este trabajo muestran la utilidad de HILIC-MS^n para separar y caracterizar mezclas complejas de GOS sin un paso previo de fraccionamiento, enriquecimiento o derivatización.

La HILIC no sólo se ha empleado de forma analítica sino también semipreparativa, permitiendo una separación eficaz de carbohidratos de distinto DP. Así, en 2009, Coulier y col. emplearon una fase estacionaria polimérica y distintos porcentajes de acetonitrilo:agua en modo isocrático para conseguir el aislamiento de GOS de DP2 y DP3, que fueron posteriormente analizados mediante HPAEC-PAD.

En 2010, Fu y col. desarrollaron un método para el análisis de distintos oligosacáridos, entre ellos GOS y FOS. Los FOS son oligosacáridos obtenidos por hidrólisis enzimática de la inulina o sintetizados con β-fructofuranosidasas usando como sustrato la sacarosa. Para la separación de estos compuestos se empleó una columna de sílice con grupos maltosa (“click” maltosa) fabricada en el laboratorio y mezclas acetonitrilo:agua como fase móvil. Para mostrar el potencial de separación del método HILIC desarrollado, se aplicó éste al análisis de mezclas complejas de FOS de alto peso molecular, consiguiéndose la correcta resolución de oligosacáridos con DP entre 10 y 50, y resolviéndose más de 40 picos en 60 minutos. Este mismo método se adaptó a condiciones semipreparativas y, como resultado, se obtuvieron distintos oligosacáridos con alta pureza en un rango de DP de 2-7. A modo de ejemplo, se muestra el perfil cromatográfico obtenido para la purificación de GOS (**Figura 6**). Este método de HILIC muestra ventajas como la buena reproducibilidad y estabilidad del procedimiento preparativo.

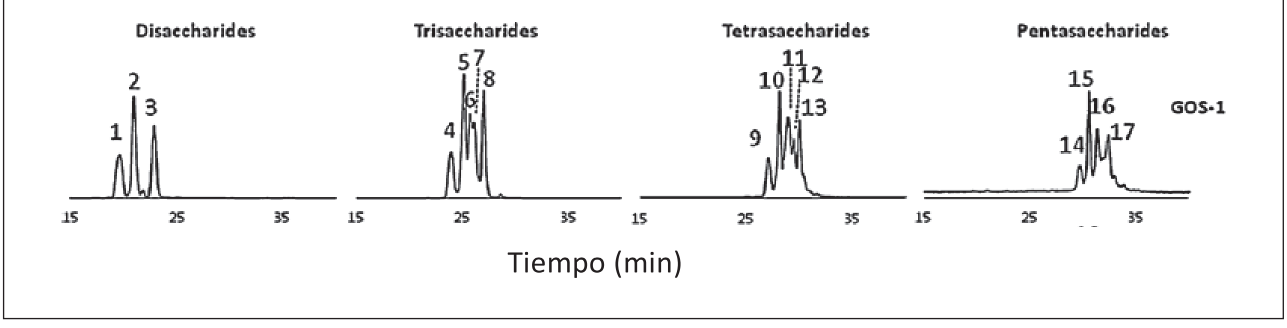


Figura 5. Perfiles de los GOS comerciales analizados por HILIC-MS^n. Reproducida con permiso de Elsevier (Hernández-Hernández y col., 2011).

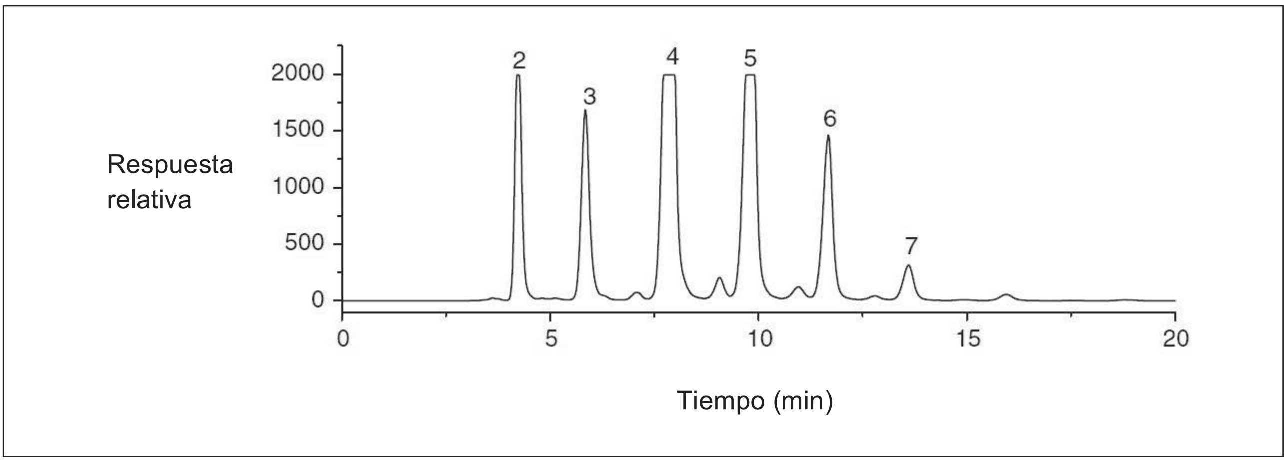


Figura 6. Cromatograma obtenido por HILIC semipreparativa para la purificación de GOS. Los números de los picos corresponden al DP de los oligosacáridos. Reproducida con permiso de Elsevier (Fu y col., 2010).

Otro de los prebióticos es la inulina, polisacárido formado por monómeros de fructosa unidos por enlaces β-(1→2) con una unidad inicial de glucosa, enlazados de forma lineal, ramificada o cíclica. Es menos estudiado que los GOS y los FOS debido a su alto peso molecular, pero aun así existen algunos trabajos (Wang y col., 2010). Los cicloinulinoligosacáridos (ciclofructanos, CF) son oligómeros de fructofuranosa ciclados producidos por la fermentación de la inulina por el *Bacillus circulans*. Wang y col. (2010) desarrollaron un método basado en el empleo de una columna β-ciclodextrina y una fase móvil hidro-orgánica con altos porcentajes de acetonitrilo para el análisis de CF y sus isómeros las ciclodextrinas. Al igual que en casos anteriores los tiempos de retención y separación decrecen a medida que aumenta el agua en la fase móvil, siendo la retención mayor cuanto mayor es el DP (**Figura 7**). Estos autores llevaron también a cabo estudios en condiciones isocráticas con distintos porcentajes de agua en la fase móvil para evaluar el mecanismo de retención de estos compuestos.

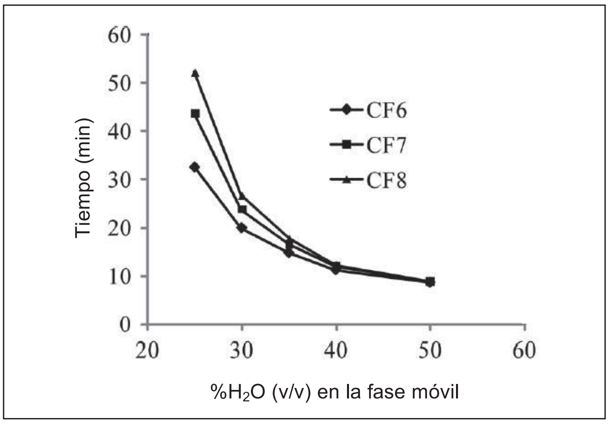


Figura 7. Tiempo de retención de CFs de distinto DP vs. porcentaje de agua en la fase móvil. Reproducida con permiso de Elsevier (Wang y col., 2010).

En cuanto a la lactulosa, Gal-β-(1→4)-Fru, disacárido sintético obtenido a través de la isomerización de la lactosa, Kubica y col. (2012) emplearon un método basado en el empleo de una columna zwitteriónica tipo sulfobetaína, y una fase móvil acuosa con acetato de amonio (5 mM), para la determinación de este disacárido y otros carbohidratos (manitol y sacarosa) en muestras de orina, con objeto de estudiar la permeabilidad de la pared intestinal. El uso de un detector de MS/MS permitió distinguir moléculas con el mismo peso molecular como lactulosa y sacarosa. A pesar de la complejidad de la matriz, el método propuesto resultó ser rápido, sencillo, sensible y reproducible.

5. CONCLUSIONES

HILIC ha demostrado ser un modo de HPLC adecuado para el análisis de carbohidratos bioactivos, tanto de alto como de bajo peso molecular, gracias a su alto poder de resolución y eficacia. Además, la posibilidad de acoplamiento directo a espectrometría de masas, principalmente en equipos con analizadores de masas tándem, aporta importante información estructural sobre los compuestos separados. Sin embargo, todavía existe un campo por explorar en la separación de carbohidratos del mismo peso molecular y diferentes enlaces glicosídicos o formas anoméricas. Es de prever, por tanto, que el desarrollo de columnas HILIC esté orientado en un futuro próximo en este sentido. Por otra parte, una mejora del conocimiento del mecanismo de retención ayudaría al desarrollo de dichas fases estacionarias. Por último, aunque el acoplamiento de HILIC a espectrómetros de masas con analizadores tipo QTOF o triple cuadrupolo (QqQ) ofrecen gran información estructural para los carbohidratos de bajo peso molecular (DP<3), son realmente los analizadores tipo IT los que podrían ayudar a ampliar el conocimiento sobre las estructuras de oligosacáridos de mayor DP.

AGRADECIMIENTOS

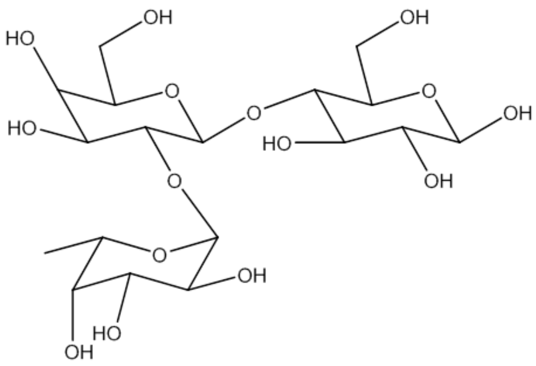
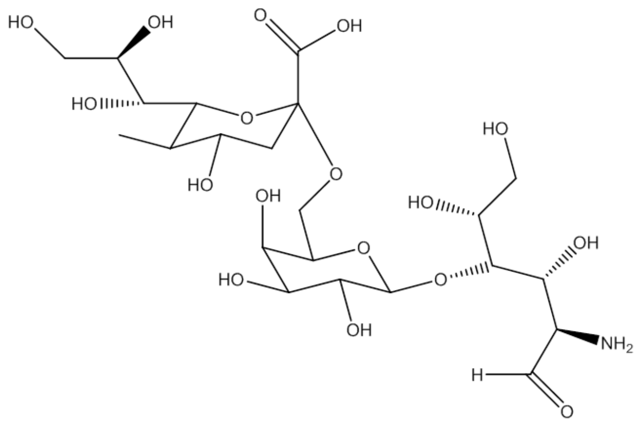
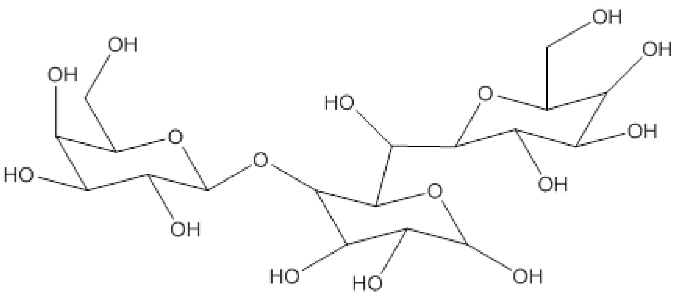
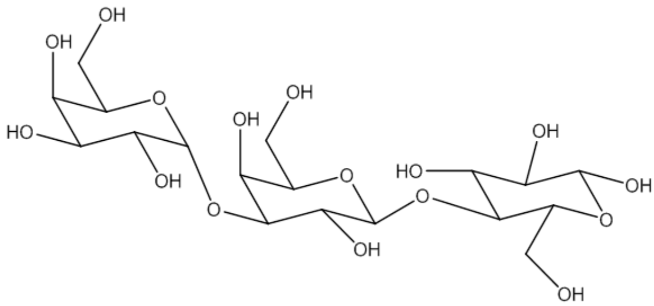
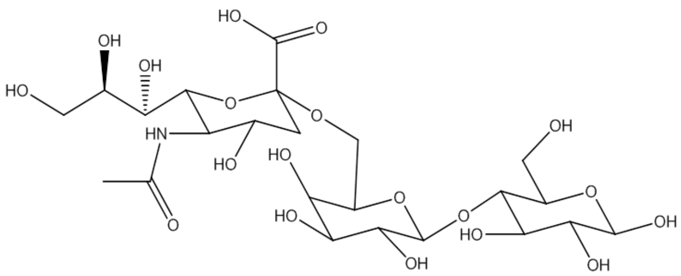
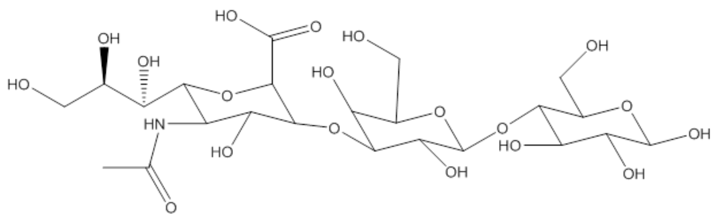
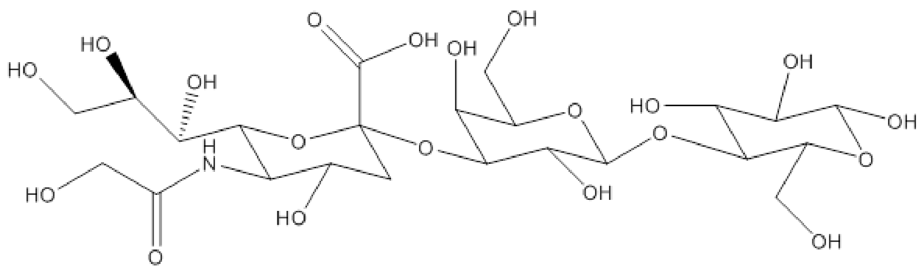
Este trabajo ha sido financiado por el Ministerio de Economía y Competitividad (proyecto CTQ2012-32957) y la Junta de Andalucía (proyecto AGR-7626). SRS agradece al Ministerio de Economía y Competitividad su beca predoctoral.

BIBLIOGRAFÍA

- Alpert, A. J., 1990. Hydrophilic-Interaction Chromatography for the Separation of peptides nucleic-acids and other polar compounds. J Chromatogr A, 499, 177-196.
- Antonio, C; Larson, T; Gilday, A; Graham, I; Bergstrom, E; Thomas-Oates, J., 2008. Hydrophilic interaction chromatography/electrospray mass spectrometry analysis of carbohydrate-related metabolites from Arabidopsis thaliana leaf tissue. Rapid Commun Mass Sp, 22, 1399-1407.
- Asano, N., Yamauchi, T., Kagamifuchi, K., Shimizu, N., Takahashi, S., Takatsuka, H., Ikeda, K., Kizu, H., Chuakul, W., Kettawan, A., Okamoto, T., 2005. Iminosugar-producing Thai medicinal plants. J Nat Prod, 68, 1238-1242.
- Brokl, M., Hernández-Hernández, O., Soria, A.C., Sanz, M.L., 2011. Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. J Chromatogr A., 1218, 7697-7703.
- Buszewski, B., Noga, S., 2012. Hydrophilic interaction liquid chromatography (HILIC) – a powerful separation technique. Anal Bioanal Chem, 402, 231-247.
- Coulier, L., Timmermans, J., Bas, R., Van Del Dool, R., Haaksman, I., Klarenbeek, B., Slahhek, T., Van Dogen, W., 2009. In-Depth Characterization of Prebiotic Galacto-oligosaccharides by a Combination of Analytical Techniques. J Agric Food Chem, 57, 8488-8495.
- Dill, K.A., 1987. The mechanism of solute retention in reversed-phase liquid-chromatography. J Phys Chem, 91, 1980-1988.
- Evans, S. V., Fellows, L. E., Shing, T. K. M., Fleet, G. W. J., 1985. Glycosidase inhibition by plant alkaloids which are structural analogs of monosaccharides. Phytochemistry, 24, 1953-1955.
- FAO Technical Meeting on prebiotics. Food and Agriculture Organization of the United Nations (FAO). Food Quality and Standards Service (AGNS). September 15-16, 2007.
- Fu, Q., Liang, T., Zhang, X., Du, Y., Guo, Z., Liang, X., 2010. Carbohydrate separation by hydrophilic interaction liquid chromatography on a 'click' maltose column. Carbohyd Res, 345, 2690-2697.
- García-Sarrió, M. J., Rodríguez-Sánchez, S., Quintanilla-López, J. E., Soria, A. C., Sanz, M. L., 2014. Analysis of iminosugars and other low molecular weight carbohydrates in *Aglaonema Commutatum Treubii* extracts by HILIC-QTOF MS. Jornadas de Análisis Instrumental, Barcelona, 2014.
- Gómez, L., Molinar-Toribio, E., Calvo-Torras, M. Á., Adelantado, C., Juan, M. E., Planas, J. M., Cañas, X., Lozano, C., Pumarola, S., Clapés, P., Torres, J. L., 2012. d-Fagomine lowers postprandial blood glucose and modulates bacterial adhesion. Brit J Nutr, 107, 1739-1746.
- Gosling, A., Stevens, G.W., Barber, A.R., Kentish, S. E., Gras, S.L., 2010. Recent advances refining galactooligosaccharide production from lactose. Food Chem, 121, 307-318.
- Greco, G., Letzel T., 2013. Main Interactions and Influences of the Chromatographic Parameters in HILIC Separations. J Chromatogr Sci, 51, 1-10.
- Guitton, J., Coste, S., Guffon-Fouilhoux, N., Cohen, S., Manchond, M., Guillaumont, M., 2009. Rapid quantification of miglustat in human plasma and cerebrospinal fluid by liquid chromatography coupled with tandem mass spectrometry. J Chromatogr B, 877, 149-154.
- Hemström, P., Irgum, K., 2006. Hydrophilic Interaction Chromatography. J Sep Sci, 29, 1784-1821.
- Hernández-Hernández, O., Calvillo, I., Lebrón-Aguilar, R., Moreno, F.J., Sanz, M.L., 2011. Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides. J Chromatogr A., 1220, 57-67.
- Ikegami, T., Horie, K., Saad, N., Hosoya, K., Fiehn, O., Tanaka, N., 2008. Highly efficient analysis of underivatized carbohydrates using monolithic-silica-based capillary hydrophilic interaction (HILIC) HPLC. Anal Bioanal Chem, 391, 2533-2542.
- Jandera, P., 2011. Stationary and mobile phases in hydrophilic interaction chromatography: a review. Anal Chim Acta, 692, 1-25.
- Kato, A., Kato, N., Miyauchi, S., Minoshima, Y., Adachi, I., Ikeda, K., Asano, N., Watson, A. A., Nash, R. J., 2008. Iminosugars from Baphia nitida Lodd. Phytochemistry, 69, 1261-1265.

- Kimura, T., Nakagawa, K., Saito, Y., Yamagishi, K., Shinmoto, H., Miyazawa, T., 2004. Simple and rapid determination of 1-deoxynojirimycin in mulberry leaves. Conferencia: 3rd International Conference on Food Factors (ICoFF-3) Tokyo, JAPAN, 2003. Biofactors. 22, 341-345.
- Kimura, T., Nakagawa, K., Saito, Y., Yamagishi, K., Suzuki, M., Yamaki, K., Shinmoto, H., Miyazawa, T., 2004. Determination of 1-deoxynojirimycin in mulberry leaves using hydrophilic interaction chromatography with evaporative light scattering detection. J Agr Food Chem, 52, 1415-1418.
- Kim, J.W., Kim, S.U., Lee, H.S., Kim, I., Ahn, M.Y., Ryu, K.S., 2003. Determination of 1-deoxynojirimycin in *Morus alba* L. leaves by derivatization with 9-fluorenylmethyl chloroformate followed by reversed-phase high-performance liquid chromatography. J Chromatogr A.,1002, 93-99.
- Knox, J.H., Pryde, A., 1975. Performance and Selected applications of a new range of chemically bonded packing materials in High-Performance Liquid-Chromatography. J Chromatogr A, 112, 171-188.
- Kubica, P., Kot-Wasik, A., Wasik, A., Namie'snik, J., Landowski, P., 2012. Modern approach for determination of lactulose, mannitol and sucrose in human urine using HPLC–MS/MS for the studies of intestinal and upper digestive tract permeability. J Chromatogr B, 907, 34-40.
- Nakagawa, K., Kubota, H., Kimura, T., Yamashita, S., Tsuzuki, T., Oikawa, S., Miyazawa, T., 2007. Occurrence of orally administered mulberry 1-deoxynojirimycin in rat plasma. J Agr Food Chem, 55, 8928-8933.
- Nakagawa, K., Ogawa, K., Higuchi, O., Kimura, T., Miyazawa, T., Hori, H., 2010. Determination of iminosugars in mulberry leaves and silkworms using hydrophilic interaction chromatography-tandem mass spectrometry. Anal Biochem, 404, 217-222.
- Nuengchamnong, N., Ingkaninan, K., Kaewruang, W., Wongareonwanakij, S., Hongthongdaeng, B., 2007. Quantitative determination of 1-deoxynojirimicycin in mulberry leaves using liquid chromatography-tandem mass spectrometry. J Pharmaceut Biomed, 44, 853-858.
- Onose, S., Ikeda, R., Nakagawa, K., Kimura, T., Yamagishi, K., Higuchi, O., Miyazawa, T., 2013. Production of the α-glycosidase inhibitor 1-deoxynojirimycin from Bacillus species. Food Chem, 138, 516-523.
- Platt, F.M., Neises, G.R., Reinkensmeier, G., Townsend, M.J., Perry, V.H., Proia, R.L., Winchester, B., Dwek, R.A., Butters, T.D., 1997. Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin. Science, 276, 428-431.
- Riedo, F., Kováts, E.S., 1982. Adsorption from Liquid-mixtures and Liquid Chromatography. J Chromatogr A 239, 1-28.
- Ritchie, G., Harvey, D. J.; Feldmann, F., Stroeher, U., Feldmann, H., Royle, L., Dwek, R.A., Rudd P.M., 2010. Identification of N-linked carbohydrates from severe acute respiratory syndrome (SARS) spike glycoprotein. Virology, 399, 257-269.
- Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.J., Leotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., Meheust, A., 2010. Prebiotic effects: metabolic and health benefits. Brit J Nutr, 104, S1-S63.
- Rodríguez-Sánchez, S., Quintanilla-López, J.E., Soria, A.C, Sanz, M.L., 2014. Evaluation of different hydrophilic stationary phases for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates in vegetable extracts by liquid chromatography tandem mass spectrometry. J Chromatogr A, 1372, 81-90.
- Sinclair, H.R., de Slegte, J., Gibson, G.R., Rastall, R.A., 2009. Galactooligosaccharides (GOS) Inhibit Vibrio cholerae Toxin Binding to Its GM1 Receptor. J Agric Food Chem, 57, 3113-3119.
- Steed, H., Macfarlane, S., 2009. Mechanisms of Prebiotic Impact on Health, en Prebiotics and Probiotics Sciences and Technology, Editores D. Charalampopoulos y R. Rastall. Springer: New York. PP 135-162.
- Tolstikov, V. V., Fiehn, O., 2002. Analysis of Highly Polar Compounds of Plant Origin: Combination of Hydrophilic Interaction Chromatography and Electrospray Ion Trap Mass Spectrometry. Anal Biochem, 301, 298-307.
- Wang, C,. Breitbach, Z. S., Armstrong, D.W., 2010. Separations of Cycloinulooligosaccharides via Hydrophilic Interaction Chromatography (HILIC) and Ligand-Exchange Chromatography. Separ Sci Technol, 45, 447-452.
- Wang, H.L., Duda, U., Radke, C.J., 1978. Solution Adsorption from Liquid Chromatography. J Colloid Interface Sci, 66, 153-165.
- Watson, A. A., Fleet, G. W. J., Asano, N., Molyneux, R. J., Nash, R. J., 2001. Polyhydroxylated alkaloids - natural occurrence and therapeutic applications. Phytoche-mistry, 56, 265-295.
- Ying, P.T., Dosey, J.G., Dill, K.A., 1989. Retention mechanisms of reversed-phase liquid-chromatography-determination of solute solvent interaction free-energies. Anal Chem, 61, 2540-2546.
- Yoshida T., 2004. Peptide separation by Hydrophilic-Interaction Chromatography: a review. J Biochem Biophys Meth, 60, 265-280.
- Yoshihashi, T., Do H.T.T.; Tungtrakul, P., Boonbumrung, S., Yamaki, K, 2010. Simple, Selective, and Rapid Quantification of 1-Deoxynojirimycin in Mulberry Leaf Products by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. J Food Sci, 75, C246-C250.

Anexo 2: Estructuras de los oligosacáridos más característicos de la leche de cabra

2'-Fucosil-lactosa	Sialil-*N*-acetil-lactosamina
	
6'-Galactosil-lactosa	3'-Galactosil-lactosa
	
6'-Sialil-lactosa	3'-Sialil-lactosa
	
Glicolil-neuraminil-lactosa	
	

Anexo 3: Correspondiente a la sección 3.1

Selective biotechnological fractionation of goat milk oligosaccharides

A. Martín-Ortiz, F.J. Moreno, A.I. Ruiz-Matute, M.L. Sanz

International Dairy Journal, 94 (2019) 38-45

Supplementary Material

Figure 1S. Concentration (mg.g^{-1}) of individual oligosaccharides during β-galactosidase treatment at short incubation times at pH 7 using 0.68 U.mL^{-1} of enzyme in pooled goat colostrum.

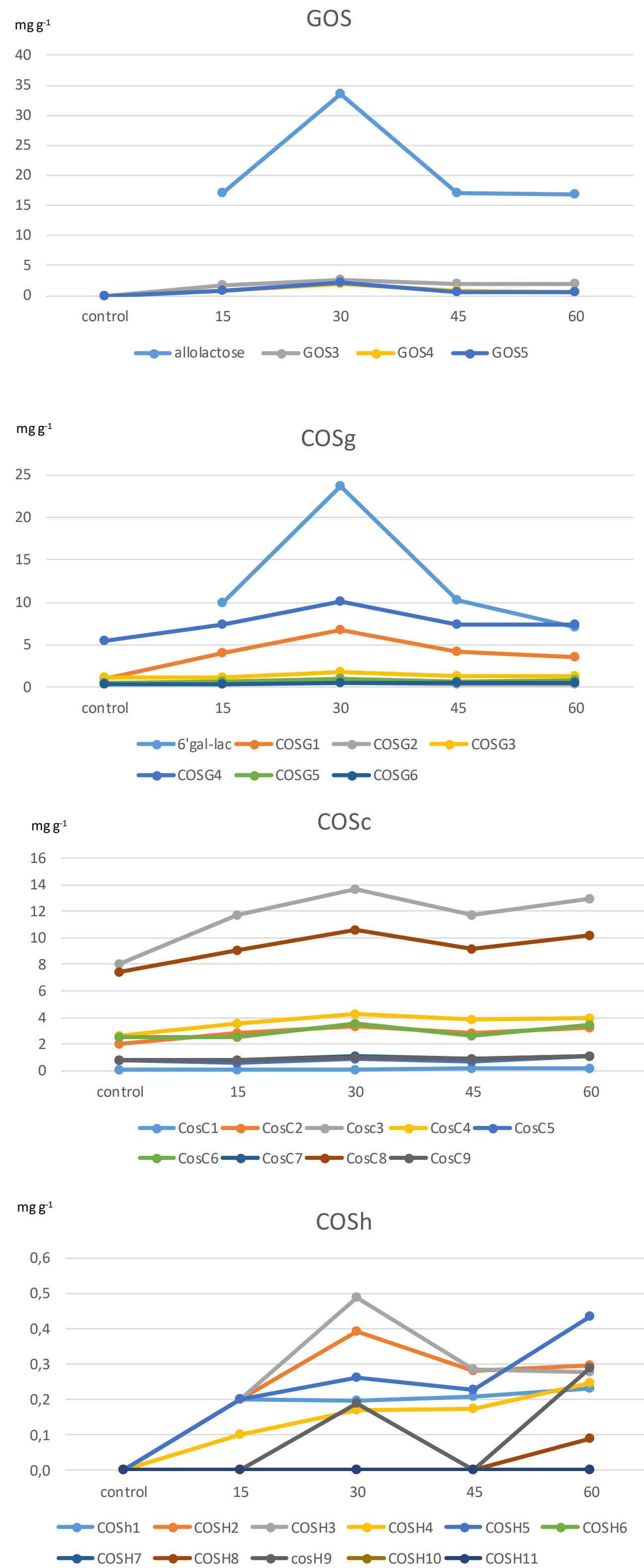
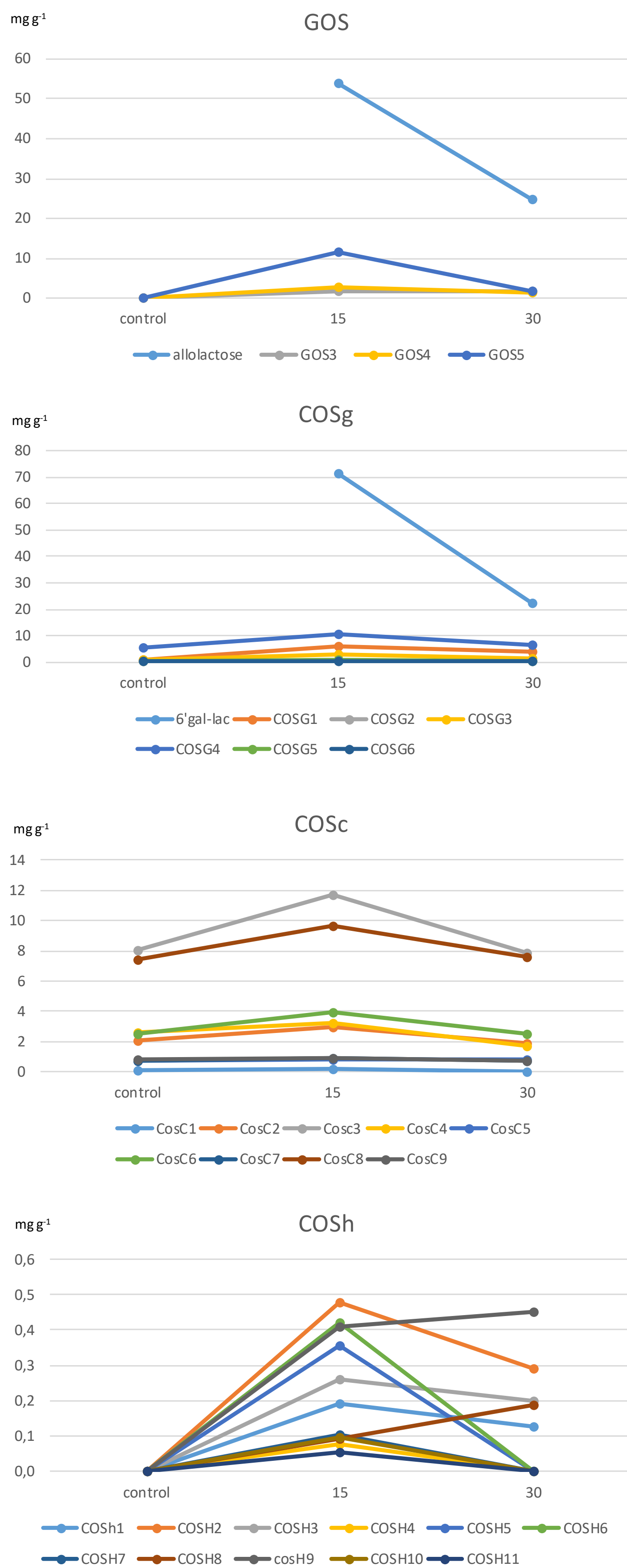


Figure 2S. Concentration (mg.g^{-1}) of individual oligosaccharide during β-galactosidase treatment at short incubation times at pH 7 using 1.02 U.mL^{-1} of enzyme in pooled goat colostrum.



Anexo 4: Correspondiente a la sección 3.3

Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry

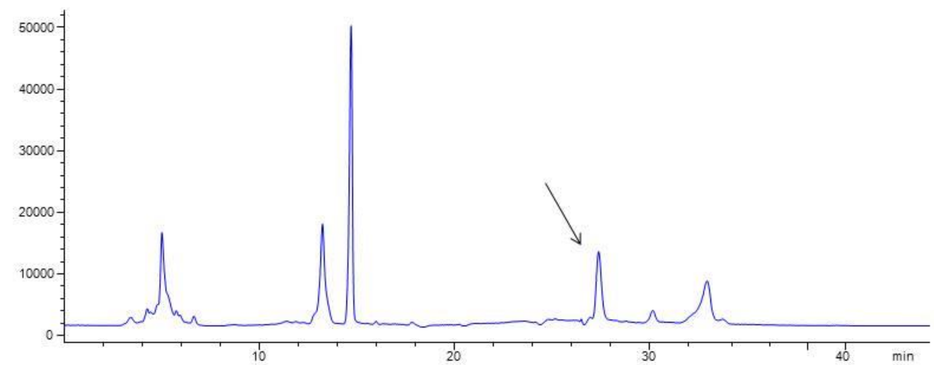
A. Martín-Ortiz, J. Salcedo, D. Barile, A. Bunyatratchata, F.J. Moreno, I. Martin-García, A. Clemente, M.L. Sanz, A.I. Ruiz-Matute

Journal of Chromatography A (Número especial), 2016, 1428, 143-153

Supplementary Material

Figure 1S. Extracted ion chromatographic profile of CS5 oligosaccharides obtained using BEH amide column and 0.1% ammonium hydroxide as mobile phase additive. A) Sample after SEC treatment (1 mg mL-1). B) Sample before treatment (0.02 mg mL-1). Peak eluting at 28 min correspond to lactose.

A)



B)

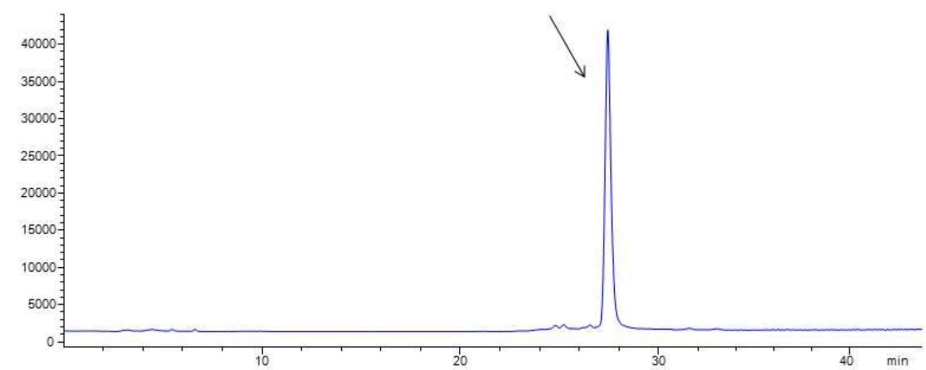
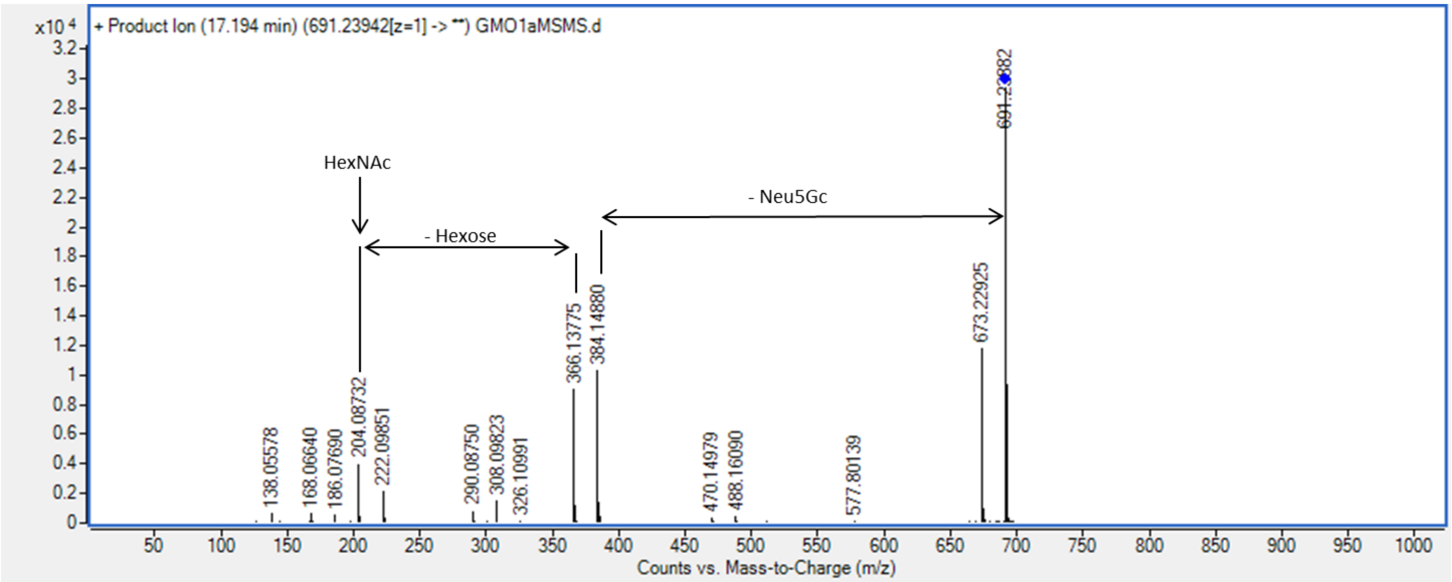
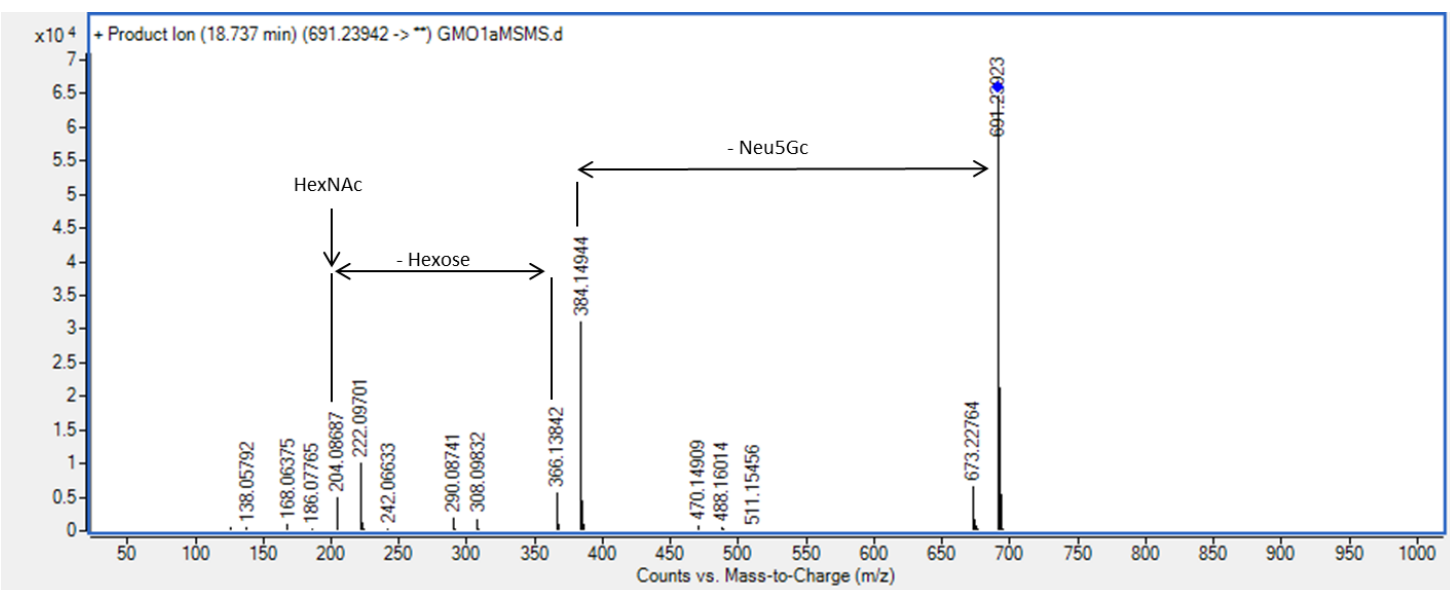
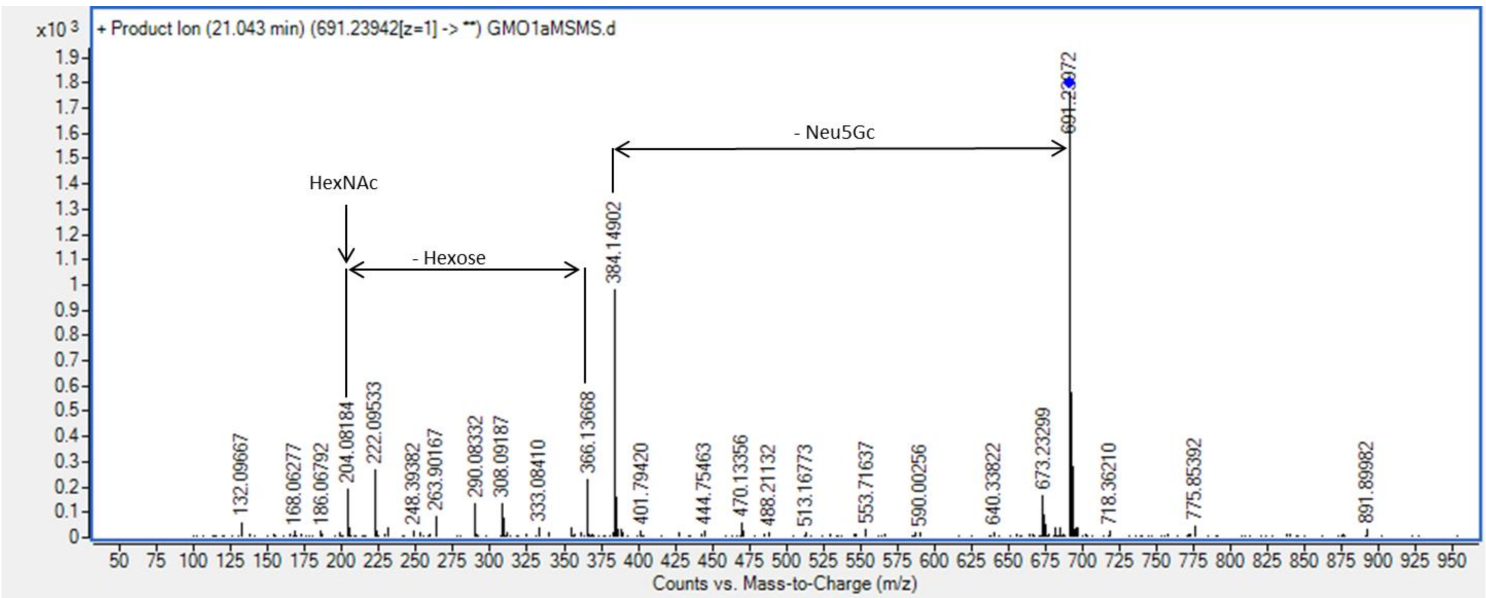


Figure 2S. MS/MS spectrum of the OS identified in the samples analyzed. OS structure is denoted as Hex_HexNAc_Fuc_Neu5Ac_Neu5Gc

a) 1_1_0_0_1



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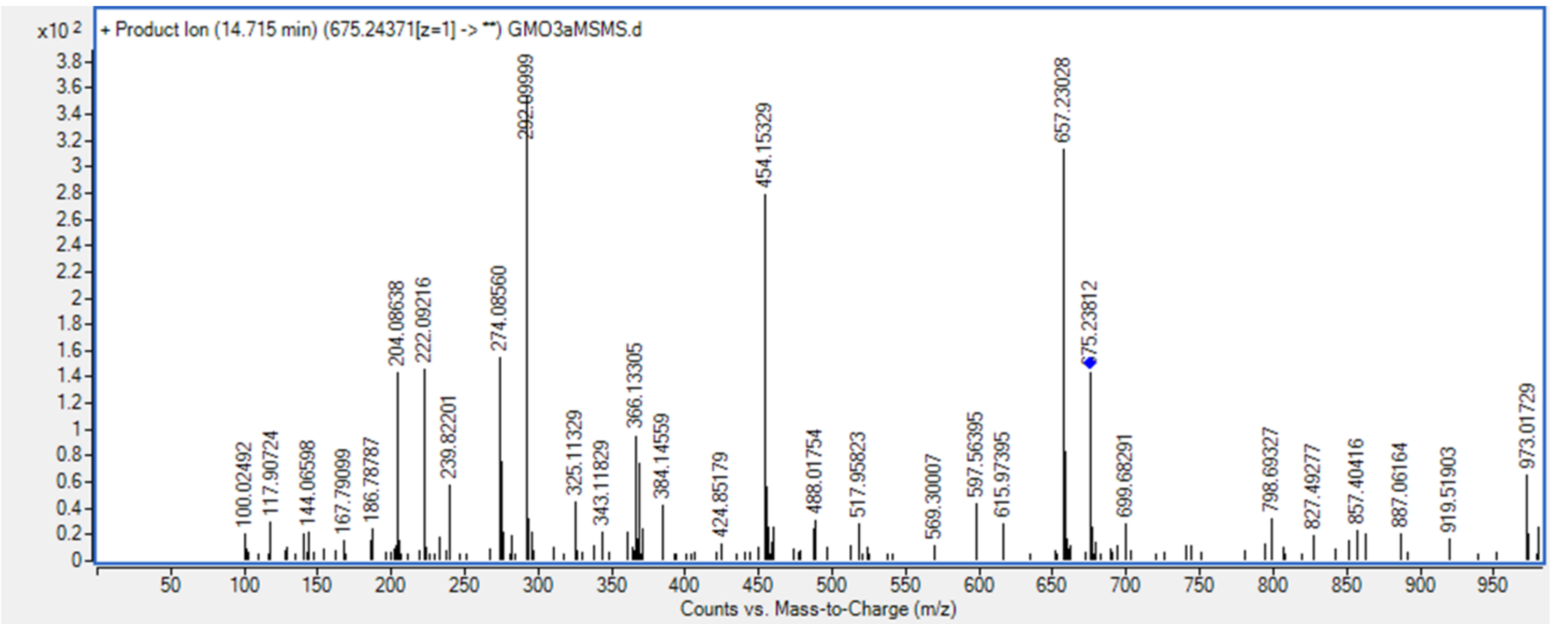
- Neu5Gc
HexNAc
- Hexose
691.23972
384.14902
366.13668
204.08184
222.09533
132.09667
168.06277
186.06792
248.39382
263.90167
290.08332
308.09187
333.08410
401.79420
444.75463
470.13356
488.21132
513.16773
553.71637
590.00256
640.33822
673.23299
718.36210
775.85392
891.89982
Counts vs. Mass-to-Charge (m/z)

b) 1_1_0_1_0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+ Product Ion (14.715 min) (675.24371[z=1] -> **) GMO3aMSMS.d
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117.90724
144.06598
167.79099
186.78787
204.08638
222.09216
239.82201
274.08560
292.09999
325.11329
343.11829
366.13305
384.14559
424.85179
454.15329
488.01754
517.95823
569.30007
597.56395
615.97395
657.23028
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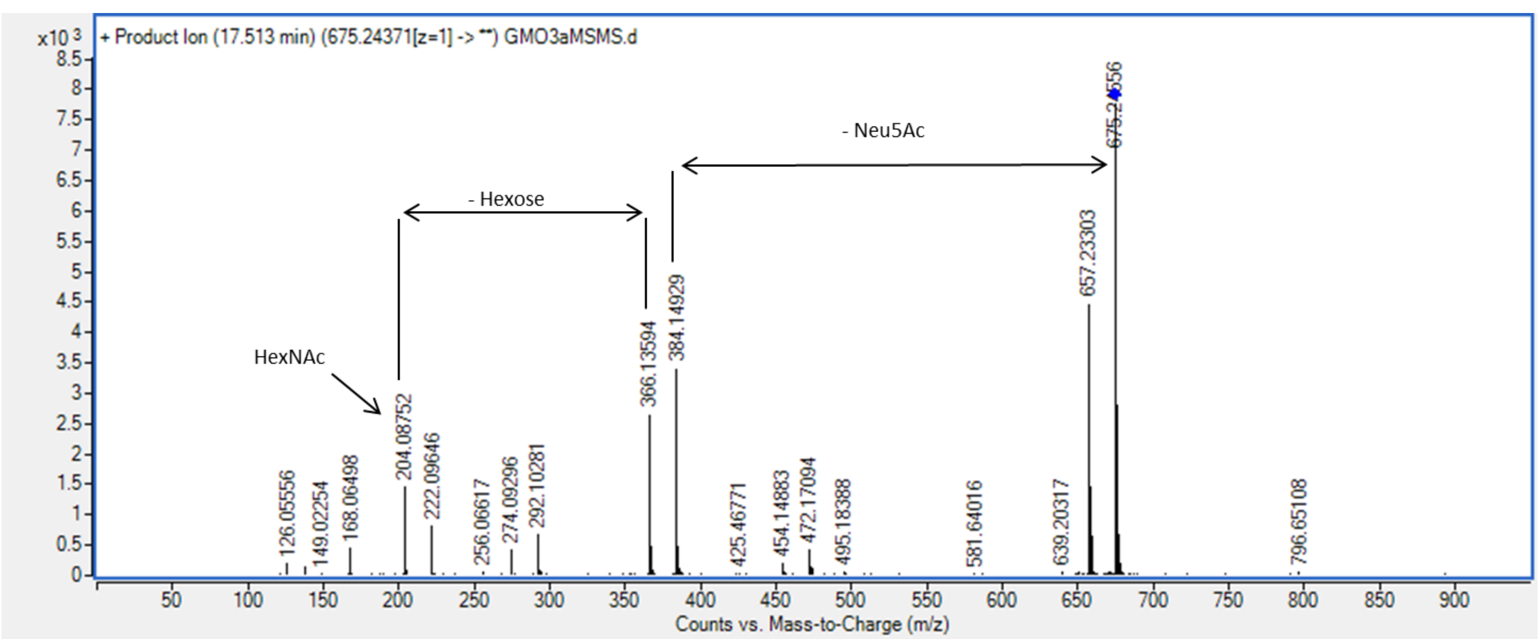
973.01729
Counts vs. Mass-to-Charge (m/z)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- Neu5Ac
- Hexose
HexNAc
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149.02254
168.06498
204.08752
222.09646
256.06617
274.09296
292.10281
366.13594
384.14929
425.46771
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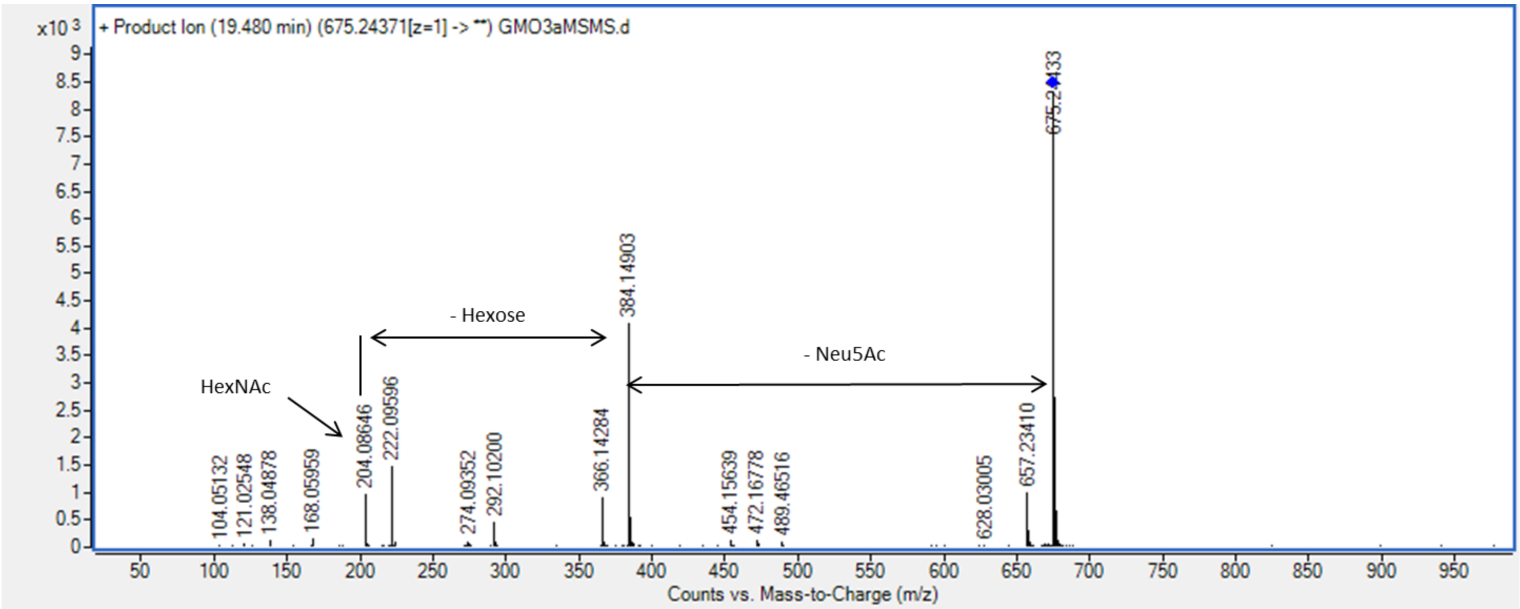
657.23303
796.65108
Counts vs. Mass-to-Charge (m/z)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+ Product Ion (19.480 min) (675.24371[z=1] -> **) GMO3aMSMS.d
675.24133
384.14903
- Hexose
- Neu5Ac
HexNAc
204.08646
222.09596
366.14284
657.23410
104.05132
121.02548
138.04878
168.05959
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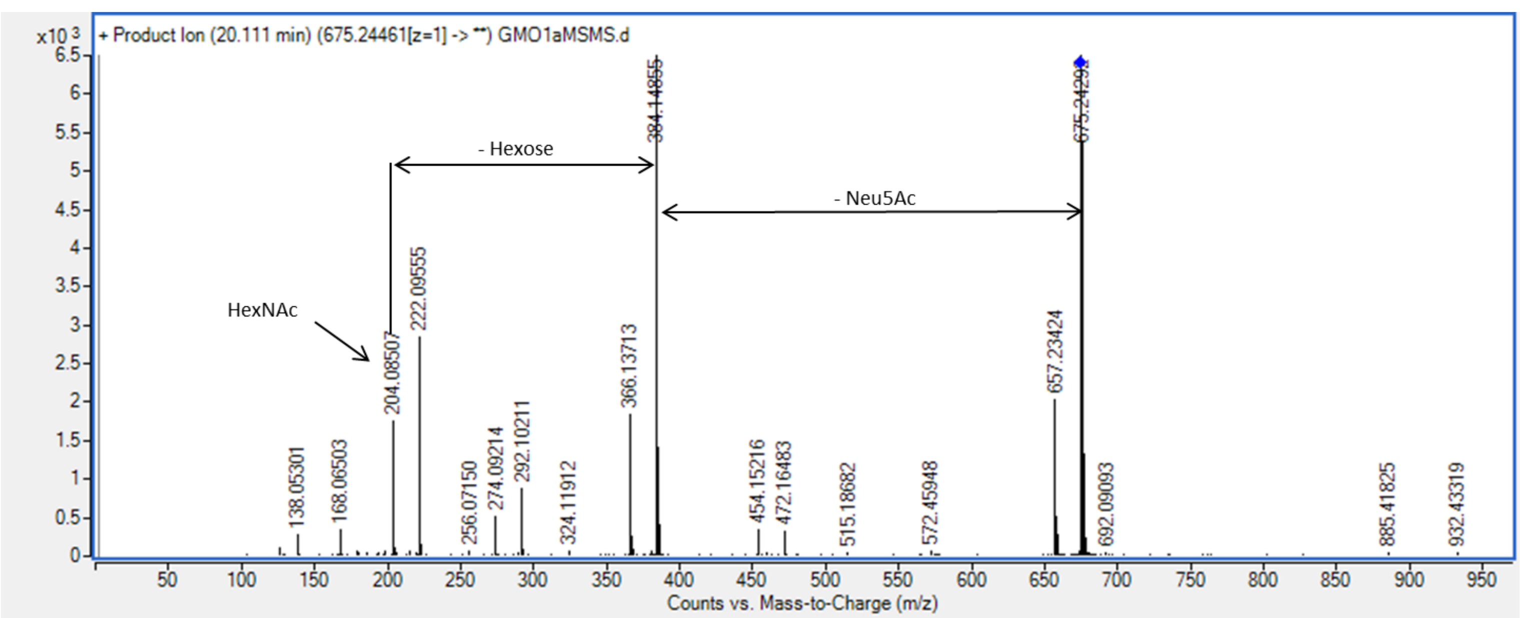
292.10200
454.15639
472.16778
489.46516
628.03005
Counts vs. Mass-to-Charge (m/z)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+ Product Ion (20.111 min) (675.24461[z=1] -> **) GMO1aMSMS.d
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675.24294
- Hexose
- Neu5Ac
222.09555
HexNAc
204.08507
366.13713
657.23424
138.05301
168.06503
256.07150
274.09214
292.10211
324.11912
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515.18682
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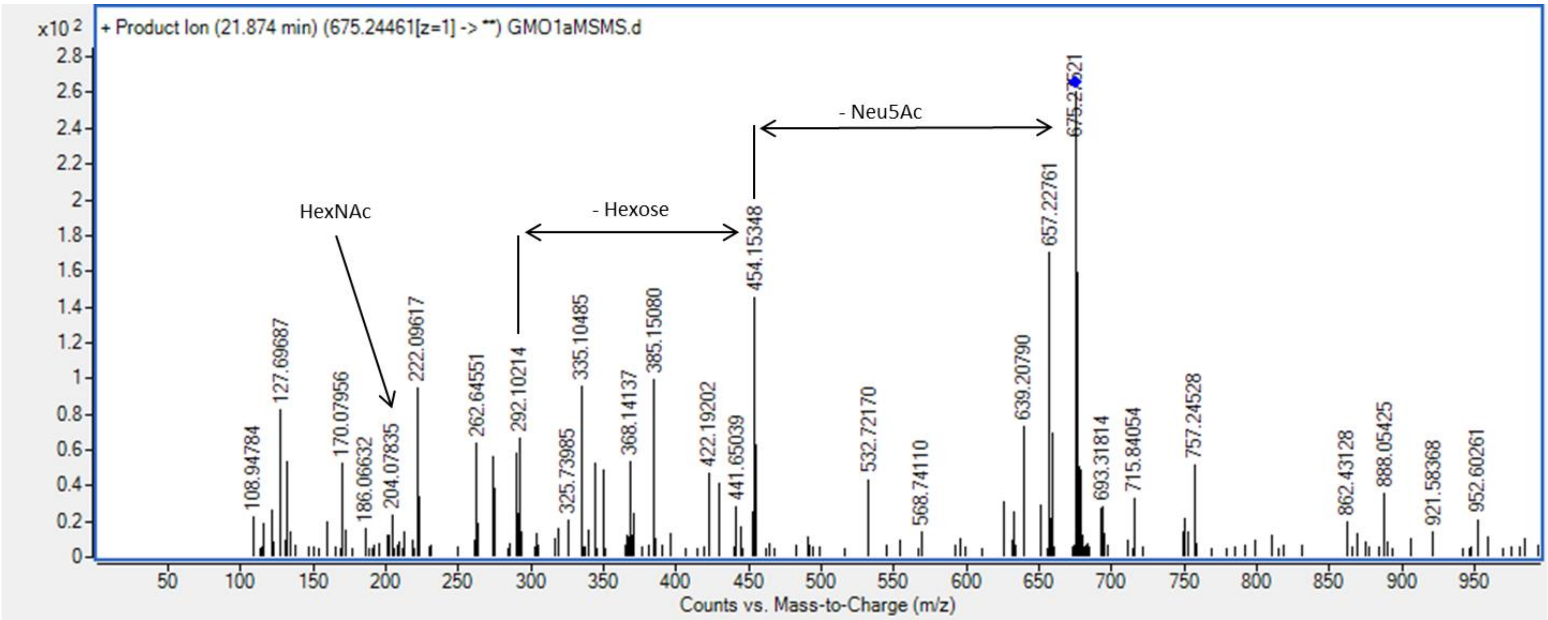
692.09093
885.41825
932.43319
Counts vs. Mass-to-Charge (m/z)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+ Product Ion (21.874 min) (675.24461[z=1] -> **) GMO1aMSMS.d
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- Hexose
HexNAc
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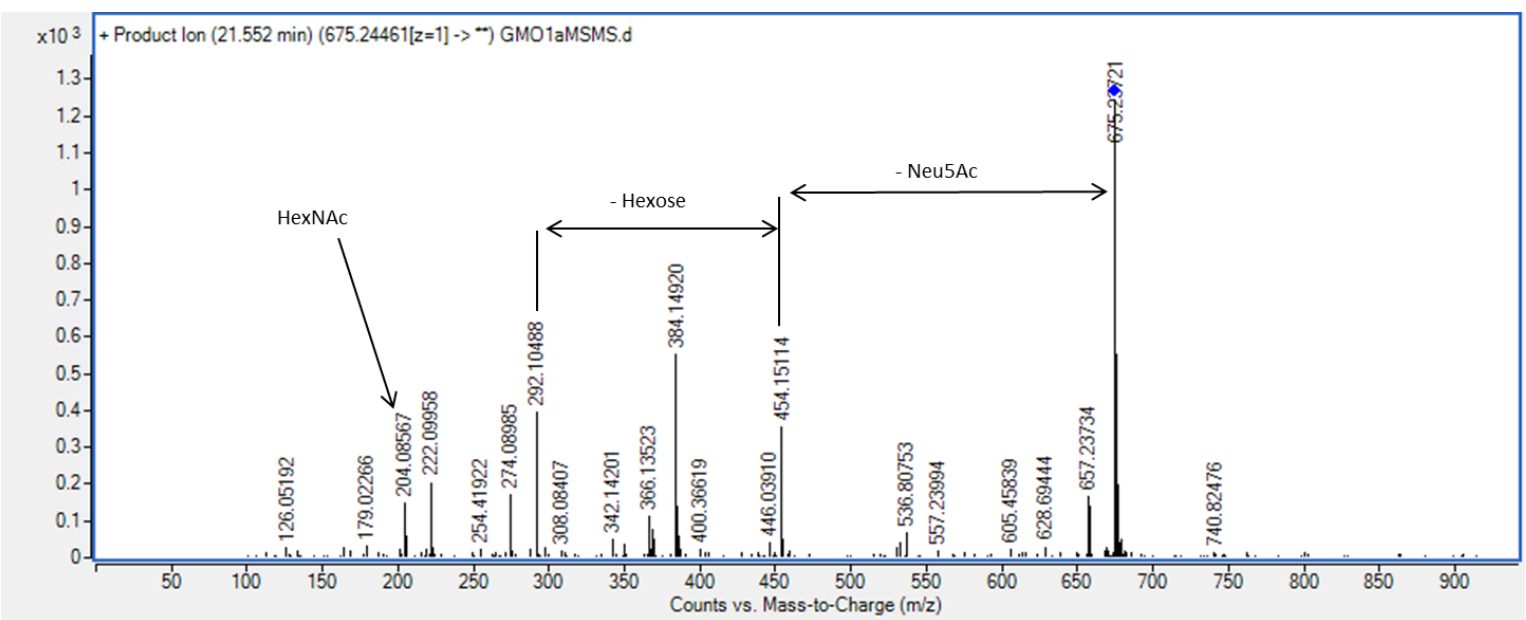
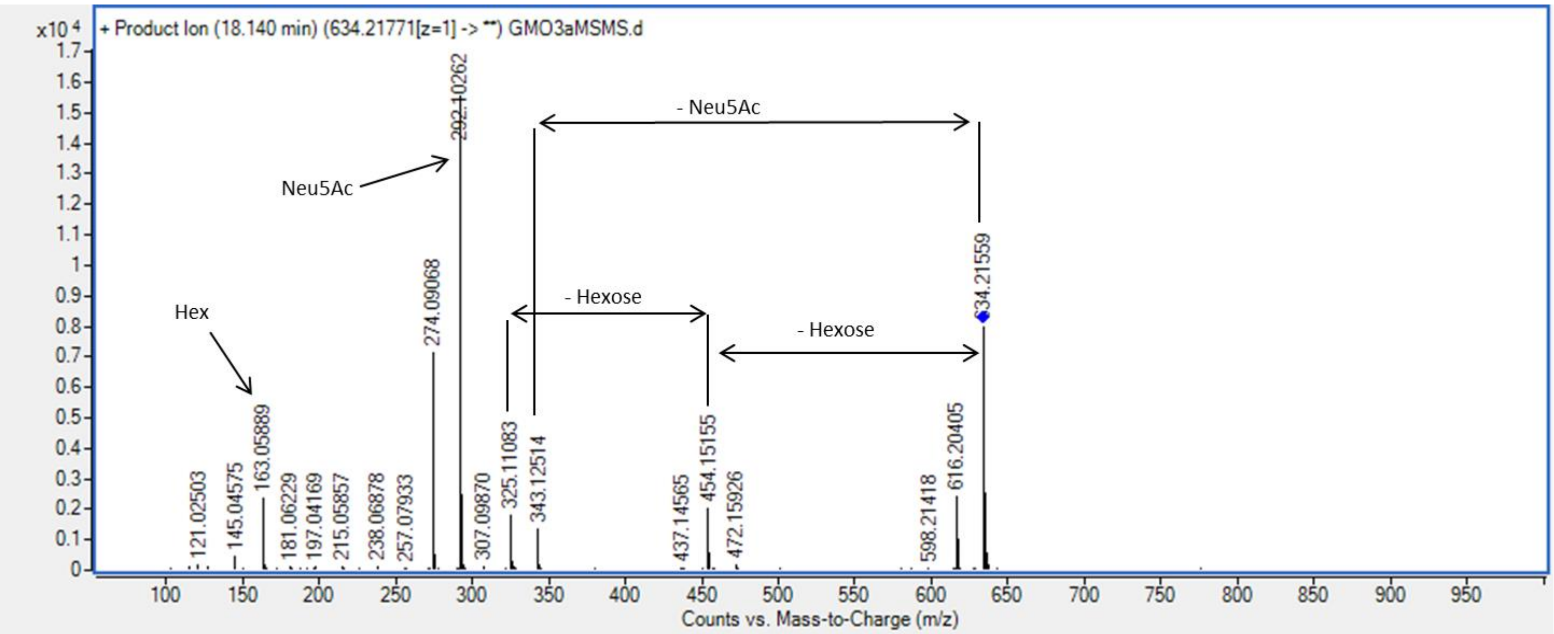
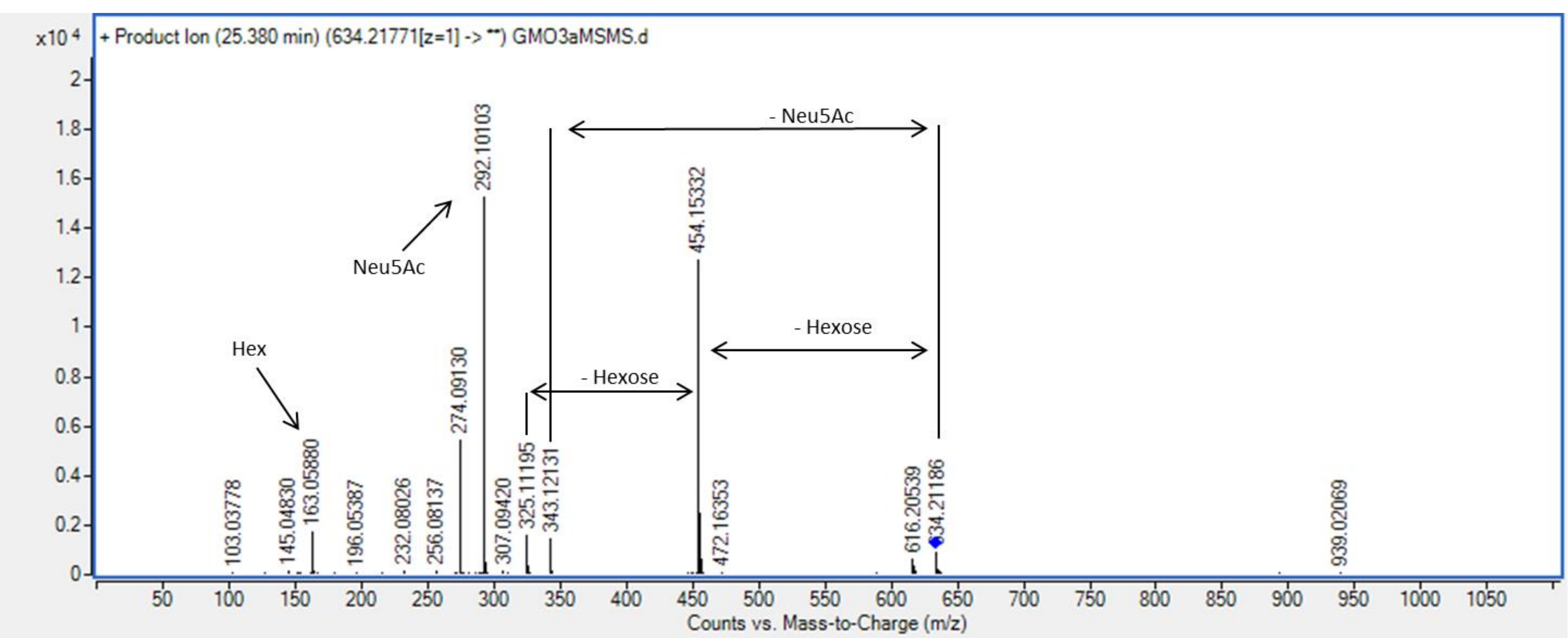
657.22761
454.15348
385.15080
335.10485
292.10214
262.64551
222.09617
127.69687
170.07956
108.94784
186.06632
204.07835
325.73985
368.14137
422.19202
441.65039
532.72170
568.74110
639.20790
693.31814
715.84054
757.24528
862.43128
888.05425
921.58368
952.60261
Counts vs. Mass-to-Charge (m/z)

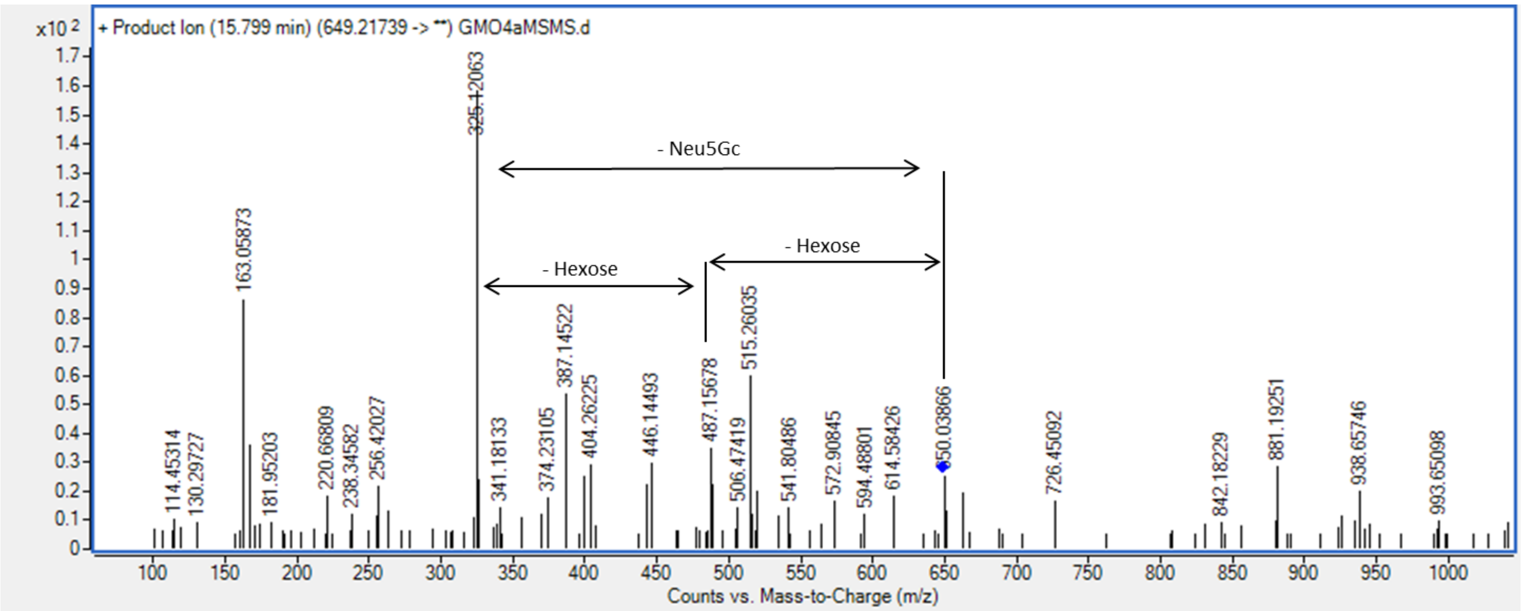

x10 3
+ Product Ion (21.552 min) (675.24461[z=1] -> **) GMO1aMSMS.d
- Neu5Ac
- Hexose
HexNAc
675.27521
384.14920
292.10488
454.15114
204.08567
222.09958
274.08985
126.05192
179.02266
254.41922
308.08407
342.14201
366.13523
400.36619
446.03910
536.80753
557.23994
605.45839
628.69444
657.23734
740.82476
Counts vs. Mass-to-Charge (m/z)

c) 2_0_0_1_0

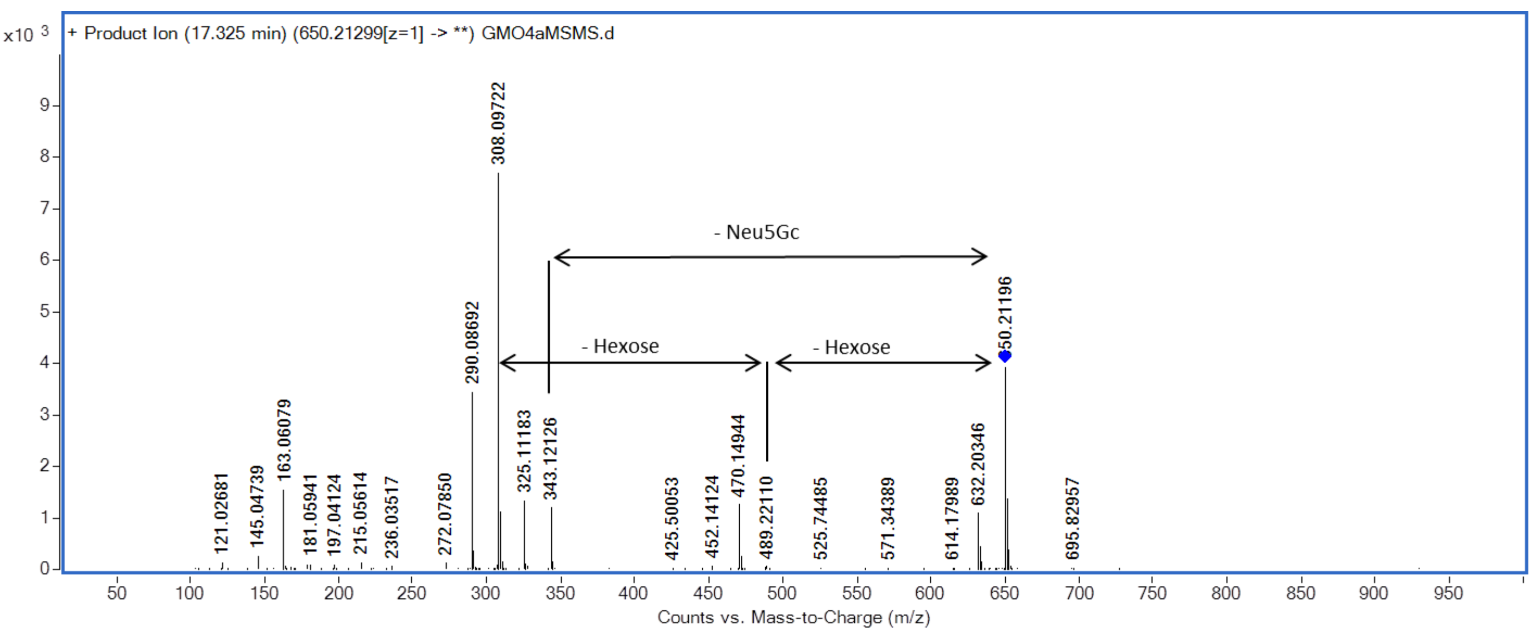

+ Product Ion (18.140 min) (634.21771[z=1] -> **) GMO3aMSMS.d
x10 4
- Neu5Ac
Neu5Ac
- Hexose
- Hexose
Hex
121.02503
145.04575
163.05889
181.06229
197.04169
215.05857
238.06878
257.07933
274.09068
292.10262
307.09870
325.11083
343.12514
437.14565
454.15155
472.15926
598.21418
616.20405
634.21559
Counts vs. Mass-to-Charge (m/z)


+ Product Ion (25.380 min) (634.21771[z=1] -> **) GMO3aMSMS.d
x10 4
- Neu5Ac
Neu5Ac
- Hexose
- Hexose
Hex
103.03778
145.04830
163.05880
196.05387
232.08026
256.08137
274.09130
292.10103
307.09420
325.11195
343.12131
454.15332
472.16353
616.20539
634.21186
939.02069
Counts vs. Mass-to-Charge (m/z)

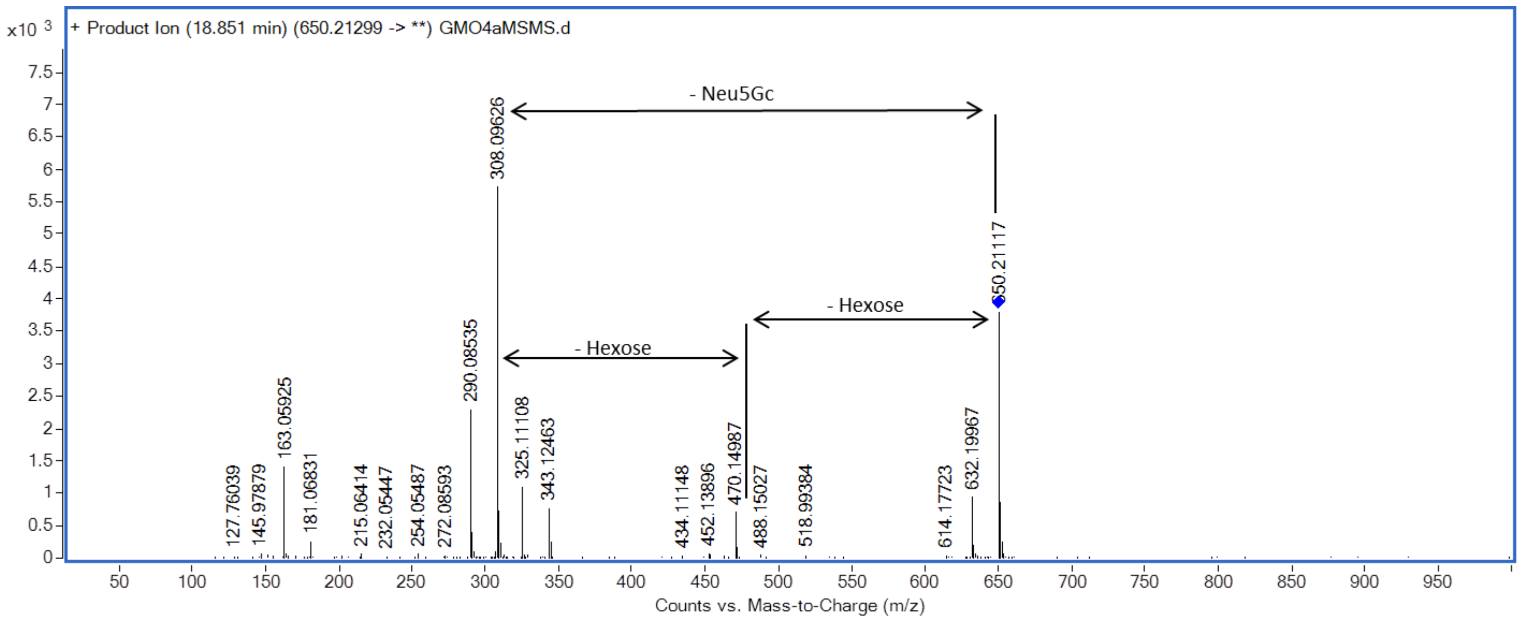
d) 2_0_0_0_1

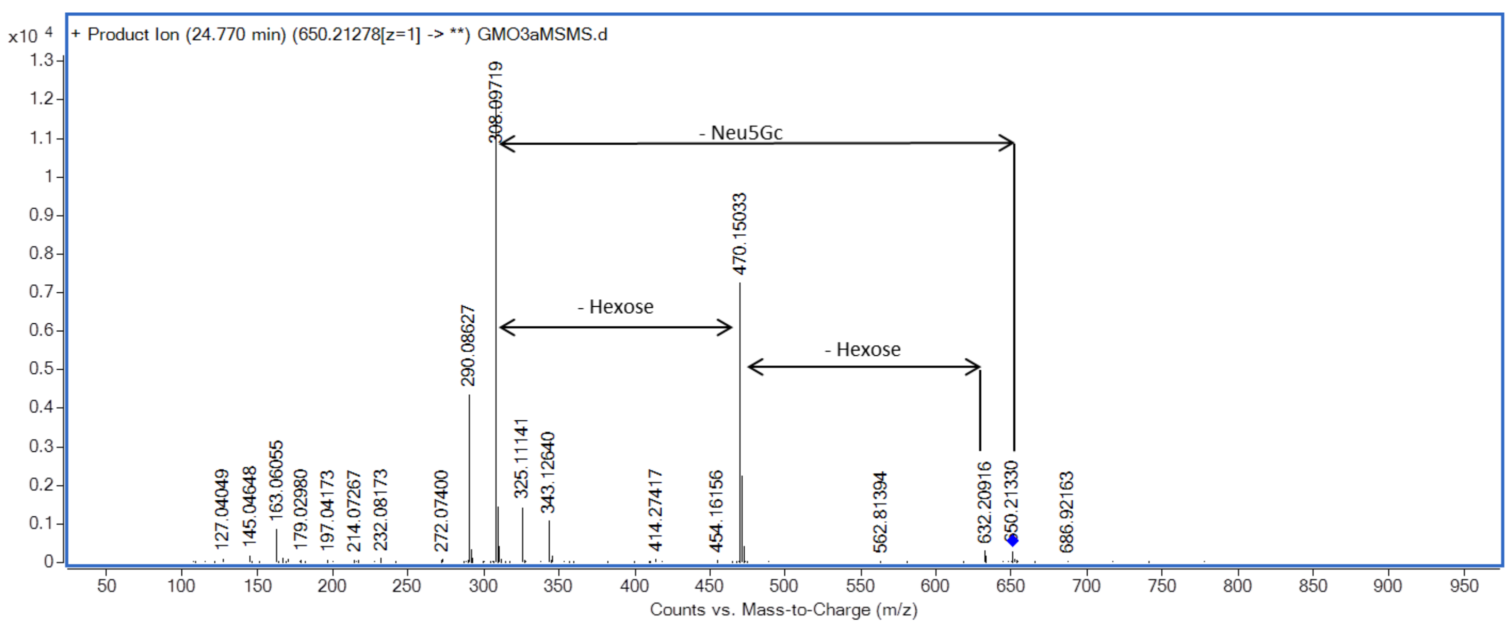


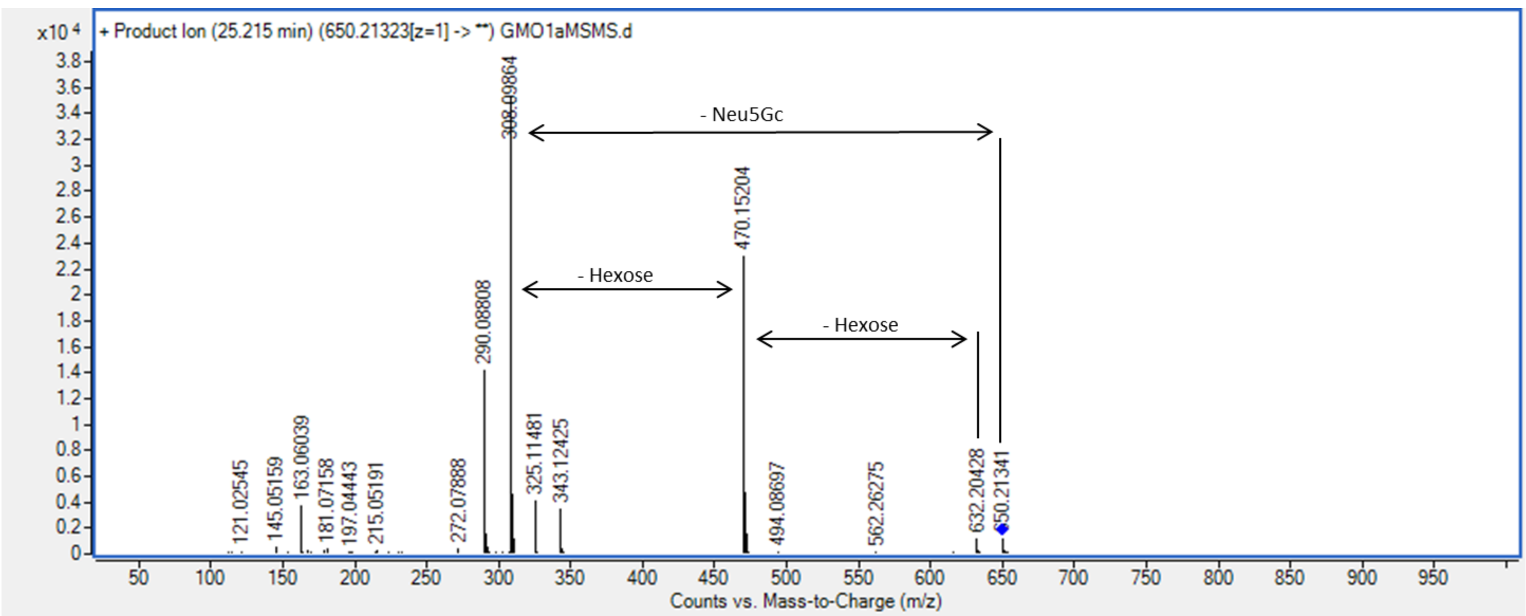
+ Product Ion (15.799 min) (649.21739 -> **) GMO4aMSMS.d
x10 2
- Neu5Gc
- Hexose
- Hexose
114.45314
130.29727
163.05873
181.95203
220.66809
238.34582
256.42027
325.12063
341.18133
374.23105
387.14522
404.26225
446.14493
487.15678
506.47419
515.26035
541.80486
572.90845
594.48801
614.58426
650.03866
726.45092
842.18229
881.19251
938.65746
993.65098
Counts vs. Mass-to-Charge (m/z)



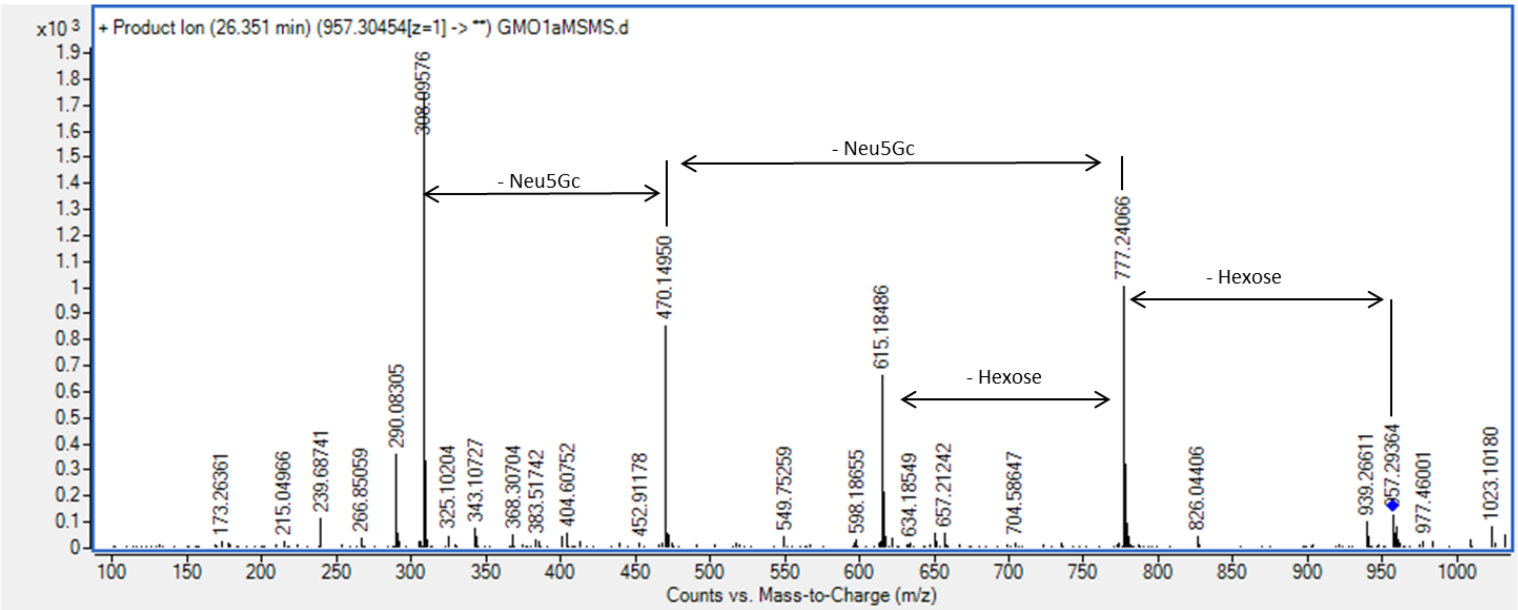
+ Product Ion (17.325 min) (650.21299[z=1] -> **) GMO4aMSMS.d
x10 3
- Neu5Gc
- Hexose
- Hexose
121.02681
145.04739
163.06079
181.05941
197.04124
215.05614
236.03517
272.07850
290.08692
308.09722
325.11183
343.12126
425.50053
452.14124
470.14944
489.22110
525.74485
571.34389
614.17989
632.20346
650.21196
695.82957
Counts vs. Mass-to-Charge (m/z)

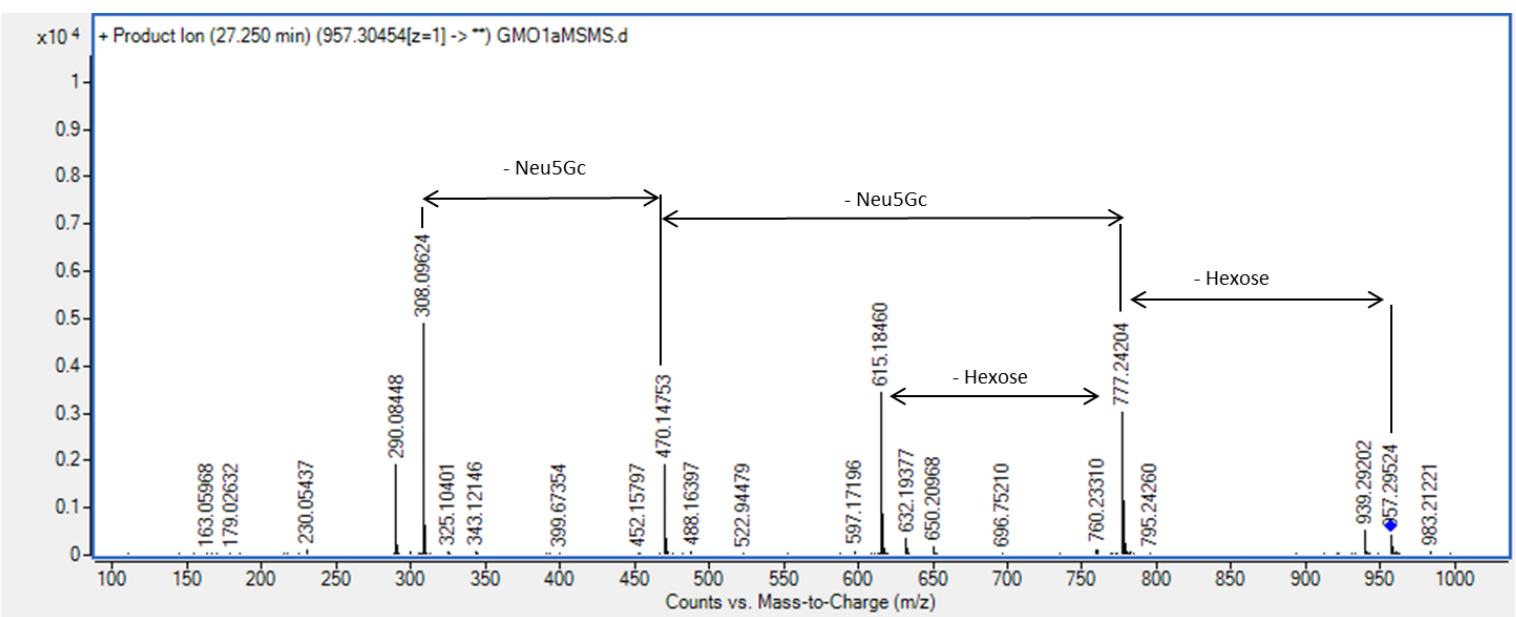

x10 3
+ Product Ion (18.851 min) (650.21299 -> **) GMO4aMSMS.d
- Neu5Gc
- Hexose
- Hexose
127.76039
145.97879
163.05925
181.06831
215.06414
232.05447
254.05487
272.08593
290.08535
308.09626
325.11108
343.12463
434.11148
452.13896
470.14987
488.15027
518.99384
614.17723
632.19967
650.21117
Counts vs. Mass-to-Charge (m/z)

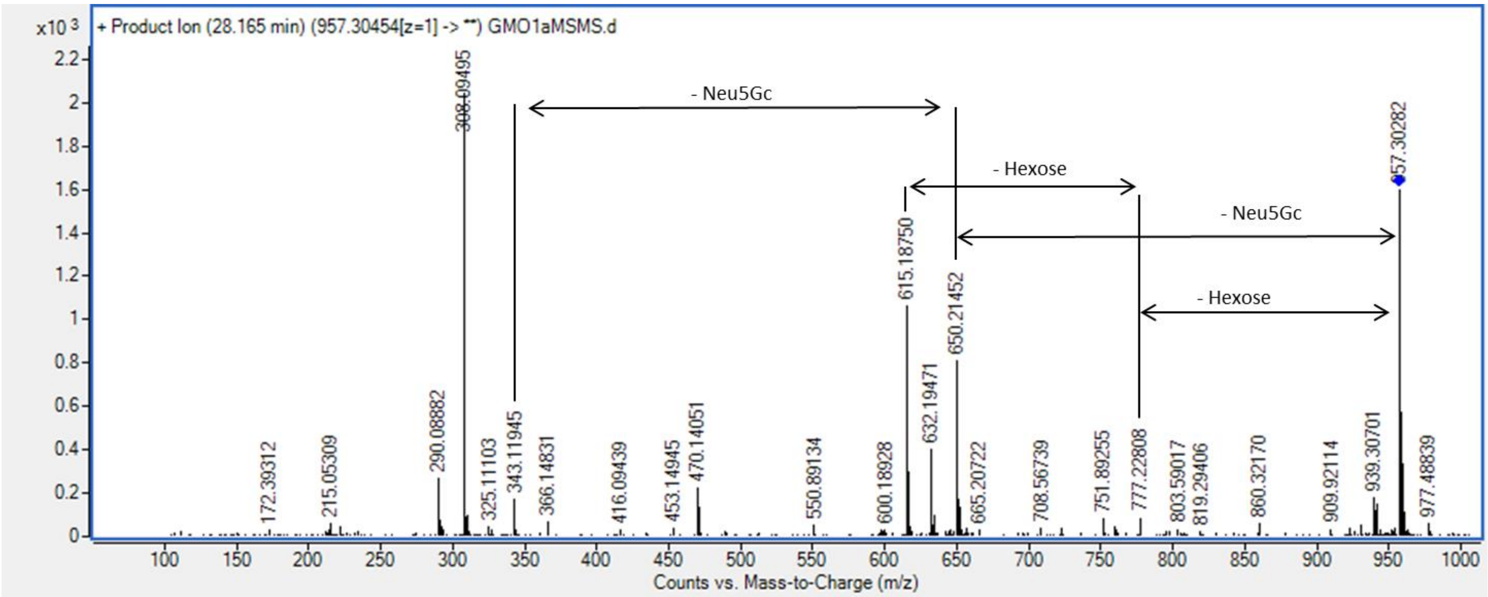

x10 4
+ Product Ion (24.770 min) (650.21278[z=1] -> **) GMO3aMSMS.d
- Neu5Gc
- Hexose
- Hexose
127.04049
145.04648
163.06055
179.02980
197.04173
214.07267
232.08173
272.07400
290.08627
308.09719
325.11141
343.12640
414.27417
454.16156
470.15033
562.81394
632.20916
650.21330
686.92163
Counts vs. Mass-to-Charge (m/z)

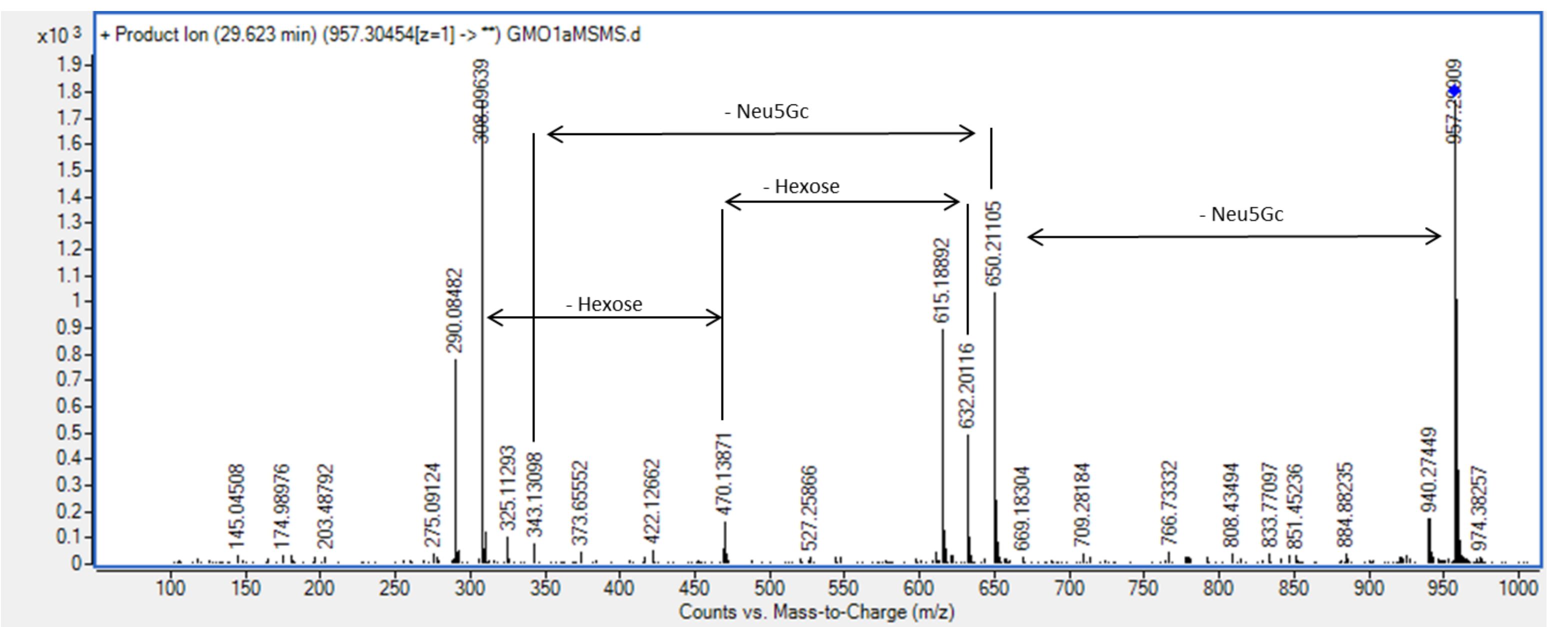

x10 4
+ Product Ion (25.215 min) (650.21323[z=1] -> **) GMO1aMSMS.d
3.8
3.6
3.4
3.2
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
308.09864
- Neu5Gc
470.15204
- Hexose
- Hexose
290.08808
121.02545
145.05159
163.06039
181.07158
197.04443
215.05191
272.07888
325.11481
343.12425
494.08697
562.26275
632.20428
650.21341
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
Counts vs. Mass-to-Charge (m/z)

e) 2_0_0_0_2

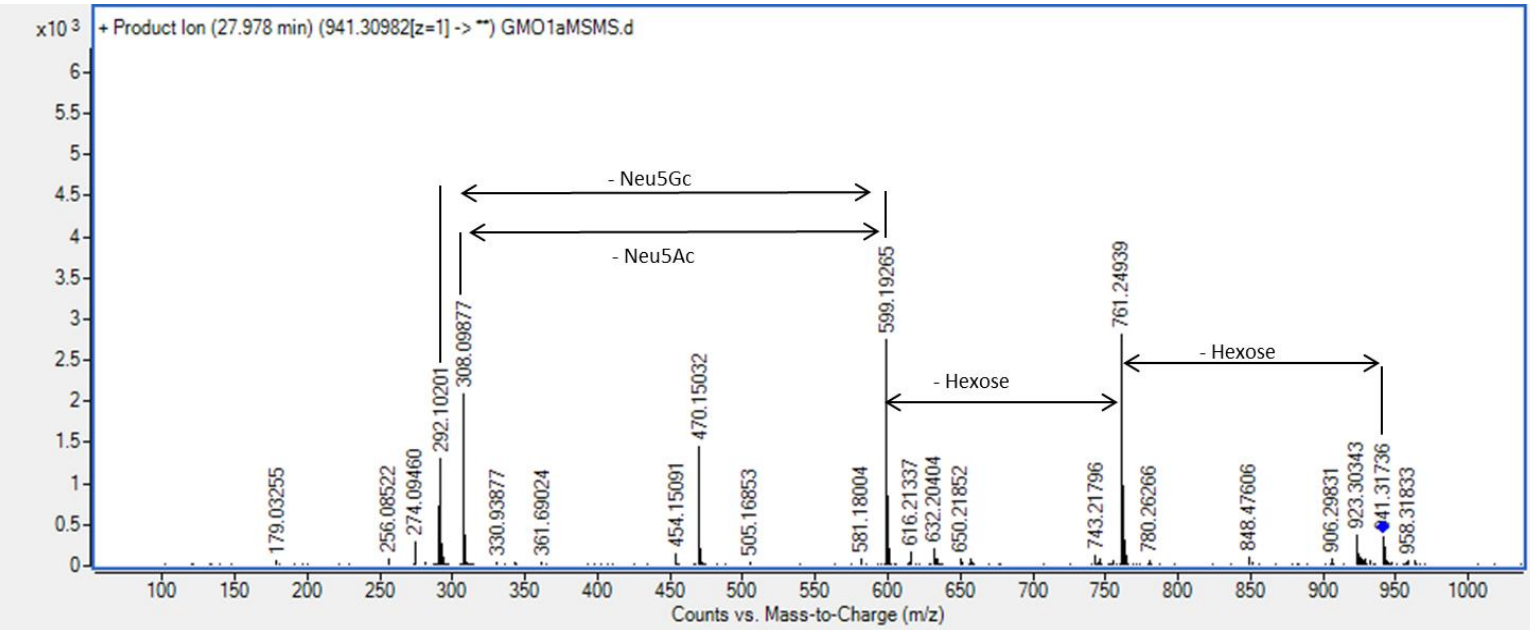

+ Product Ion (26.351 min) (957.30454[z=1] -> **) GMO1aMSMS.d
x10 3
308.09576
470.14950
777.24066
615.18486
290.08305
- Neu5Gc
- Neu5Gc
- Hexose
- Hexose
173.26361
215.04966
239.68741
266.85059
325.10204
343.10727
368.30704
383.51742
404.60752
452.91178
549.75259
598.18655
634.18549
657.21242
704.58647
826.04406
939.26611
957.29364
977.46001
1023.10180
Counts vs. Mass-to-Charge (m/z)

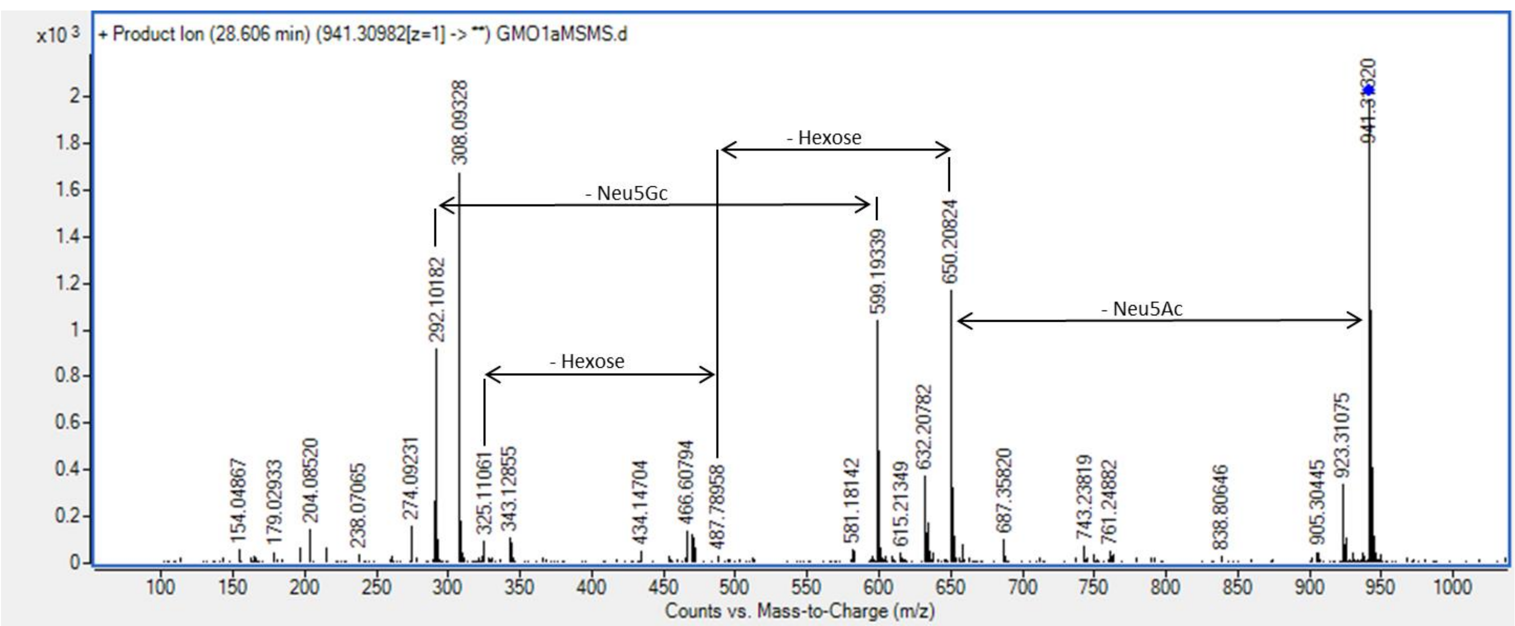

+ Product Ion (27.250 min) (957.30454[z=1] -> **) GMO1aMSMS.d
x10 4
- Neu5Gc
- Neu5Gc
- Hexose
- Hexose
308.09624
470.14753
615.18460
777.24204
290.08448
163.05968
179.02632
230.05437
325.10401
343.12146
399.67354
452.15797
488.16397
522.94479
597.17196
632.19377
650.20968
696.75210
760.23310
795.24260
939.29202
957.29524
983.21221
Counts vs. Mass-to-Charge (m/z)

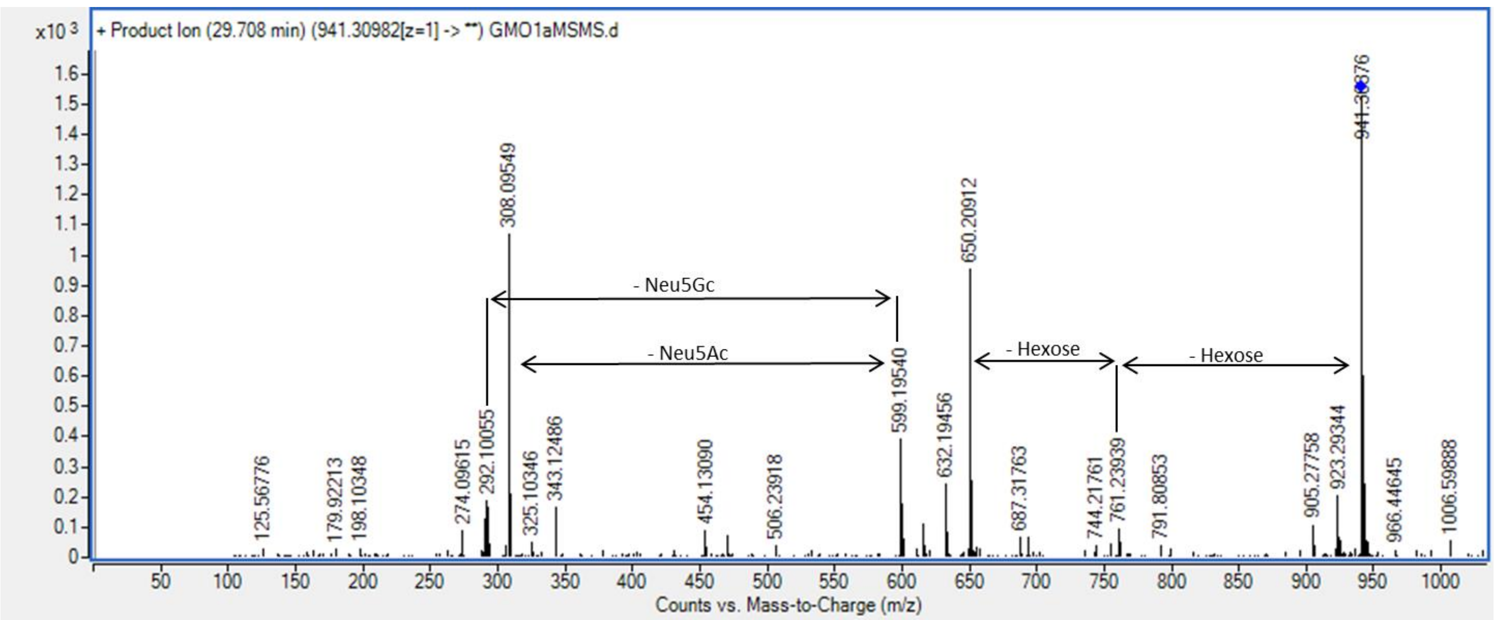

x10 3
+ Product Ion (28.165 min) (957.30454[z=1] -> **) GMO1aMSMS.d
- Neu5Gc
- Hexose
- Neu5Gc
- Hexose
308.09495
957.30282
615.18750
650.21452
632.19471
290.08882
343.11945
470.14051
777.22808
939.30701
Counts vs. Mass-to-Charge (m/z)


x10 3
+ Product Ion (29.623 min) (957.30454[z=1] -> **) GMO1aMSMS.d
- Neu5Gc
- Hexose
- Neu5Gc
- Hexose
308.09639
615.18892
650.21105
632.20116
290.08482
343.13098
470.13871
940.27449
Counts vs. Mass-to-Charge (m/z)

f) 2_0_0_1_1

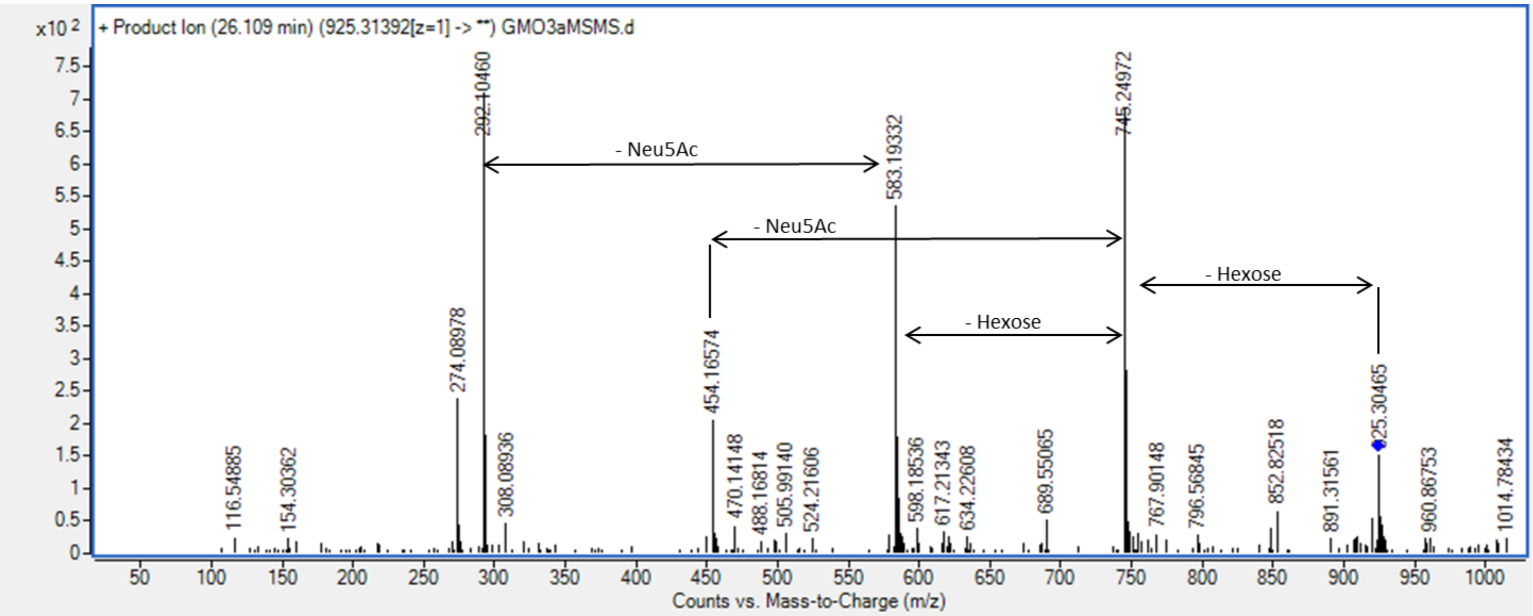

+ Product Ion (27.978 min) (941.30982[z=1] -> **) GMO1aMSMS.d
x10 3
- Neu5Gc
- Neu5Ac
- Hexose
- Hexose
179.03255
256.08522
274.09460
292.10201
308.09877
330.93877
361.69024
454.15091
470.15032
505.16853
581.18004
599.19265
616.21337
632.20404
650.21852
743.21796
761.24939
780.26266
848.47606
906.29831
923.30343
941.31736
958.31833
Counts vs. Mass-to-Charge (m/z)

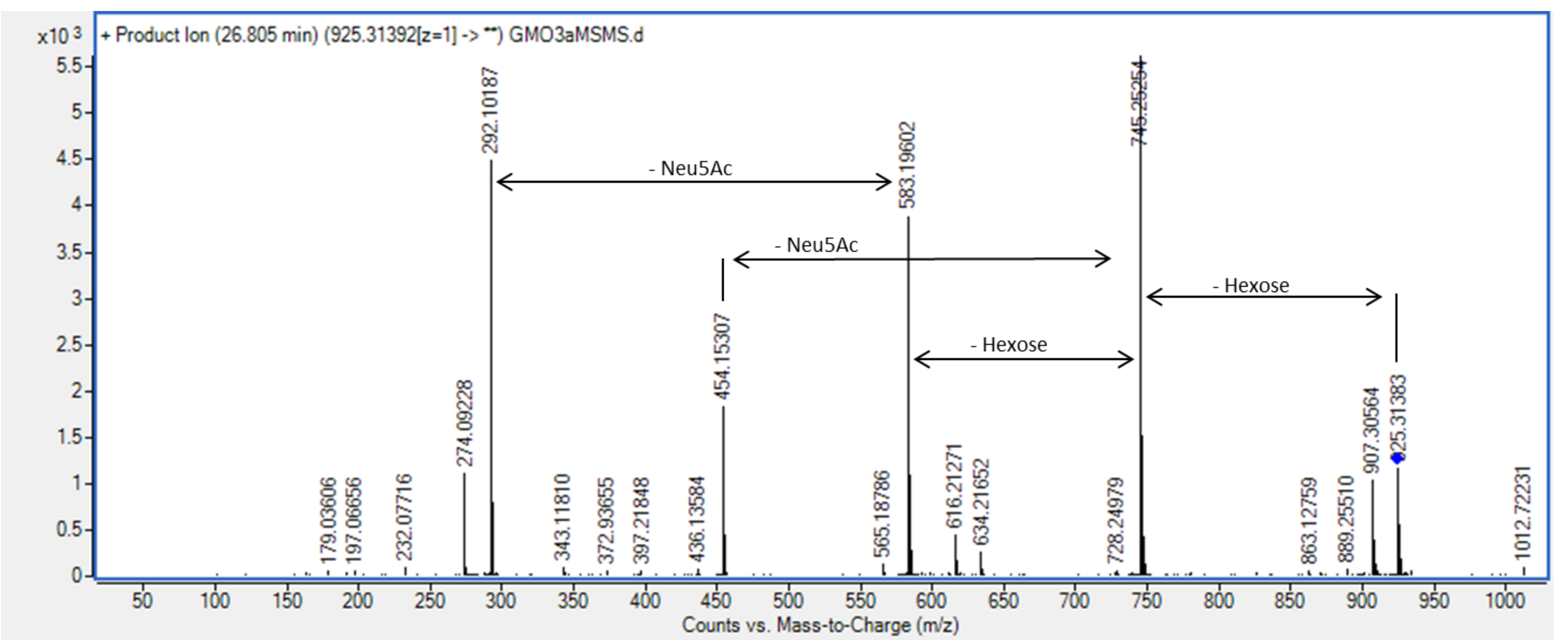

+ Product Ion (28.606 min) (941.30982[z=1] -> **) GMO1aMSMS.d
x10 3
- Hexose
- Neu5Gc
- Neu5Ac
- Hexose
154.04867
179.02933
204.08520
238.07065
274.09231
292.10182
308.09328
325.11061
343.12855
434.14704
466.60794
487.78958
581.18142
599.19339
615.21349
632.20782
650.20824
687.35820
743.23819
761.24882
838.80646
905.30445
923.31075
Counts vs. Mass-to-Charge (m/z)

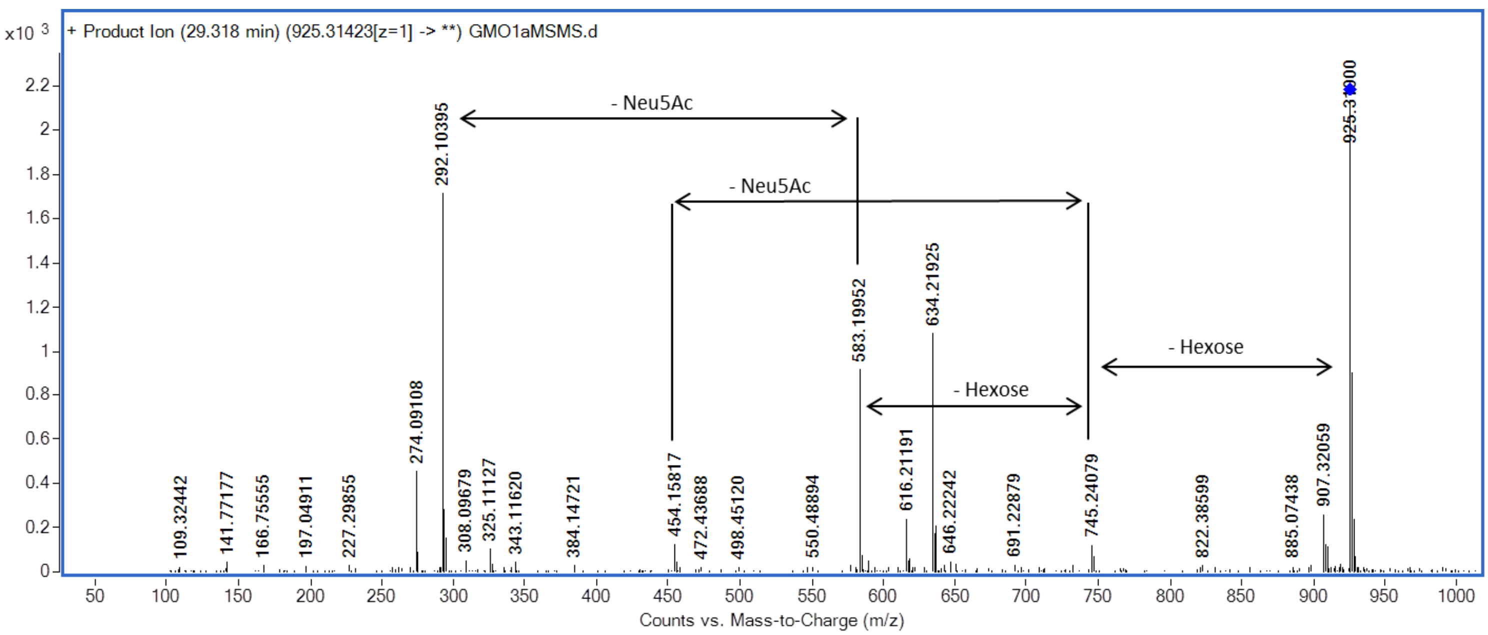


x10 3
+ Product Ion (29.708 min) (941.30982[z=1] -> **) GMO1aMSMS.d
- Neu5Gc
- Neu5Ac
- Hexose
- Hexose
125.56776
179.92213
198.10348
274.09615
292.10055
308.09549
325.10346
343.12486
454.13090
506.23918
599.19540
632.19456
650.20912
687.31763
744.21761
761.23939
791.80853
905.27758
923.29344
941.30876
966.44645
1006.59888
Counts vs. Mass-to-Charge (m/z)

g) 2_0_0_2_0

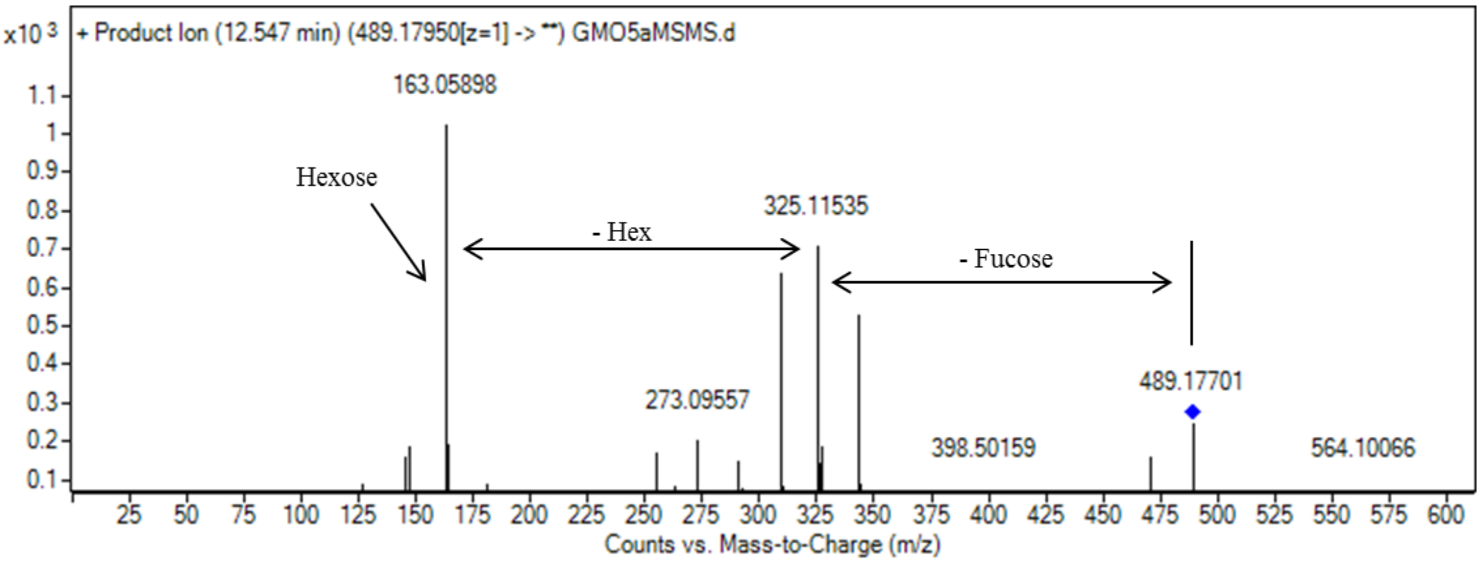

x10 2
+ Product Ion (26.109 min) (925.31392[z=1] -> **) GMO3aMSMS.d
292.10460
745.24972
583.19332
454.16574
274.08978
308.08936
116.54885
154.30362
470.14148
488.16814
505.99140
524.21606
598.18536
617.21343
634.22608
689.55065
767.90148
796.56845
852.82518
891.31561
925.30465
960.86753
1014.78434
- Neu5Ac
- Neu5Ac
- Hexose
- Hexose
Counts vs. Mass-to-Charge (m/z)


x10 3
+ Product Ion (26.805 min) (925.31392[z=1] -> **) GMO3aMSMS.d
292.10187
745.25254
583.19602
454.15307
274.09228
179.03606
197.06656
232.07716
343.11810
372.93655
397.21848
436.13584
565.18786
616.21271
634.21652
728.24979
863.12759
889.25510
907.30564
925.31383
1012.72231
- Neu5Ac
- Neu5Ac
- Hexose
- Hexose
Counts vs. Mass-to-Charge (m/z)

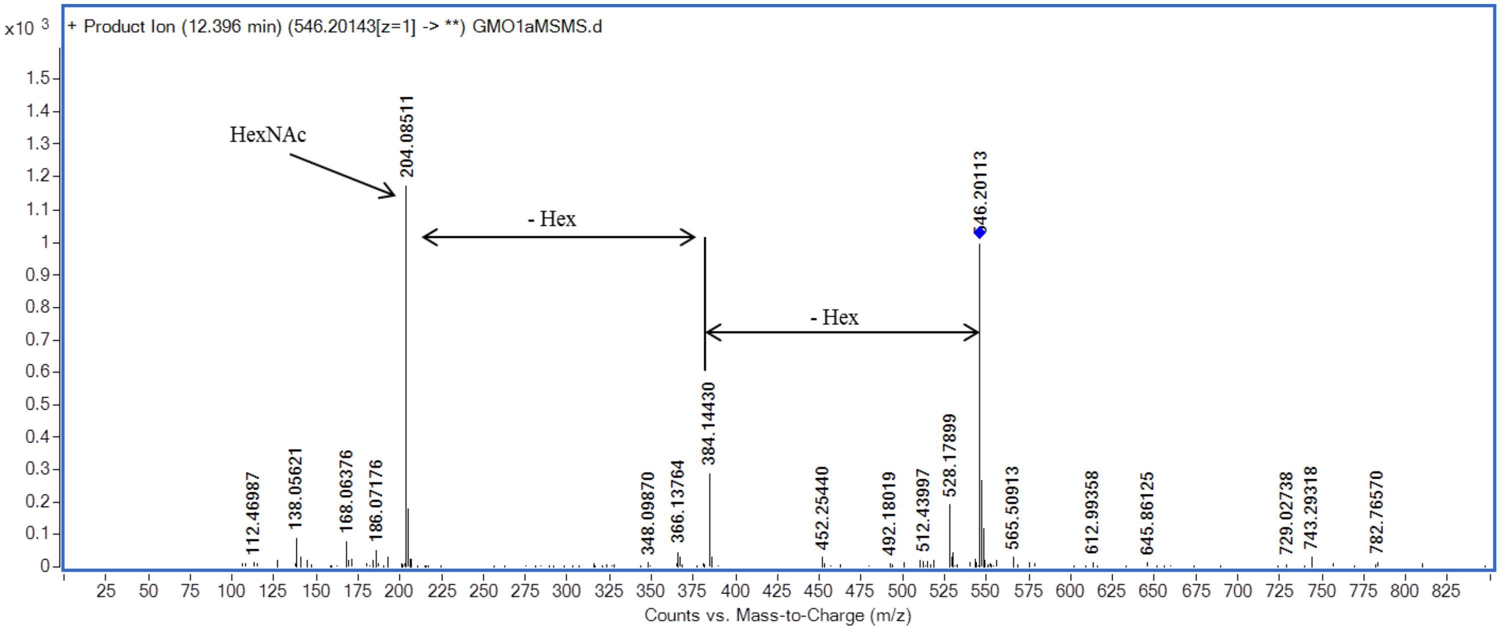


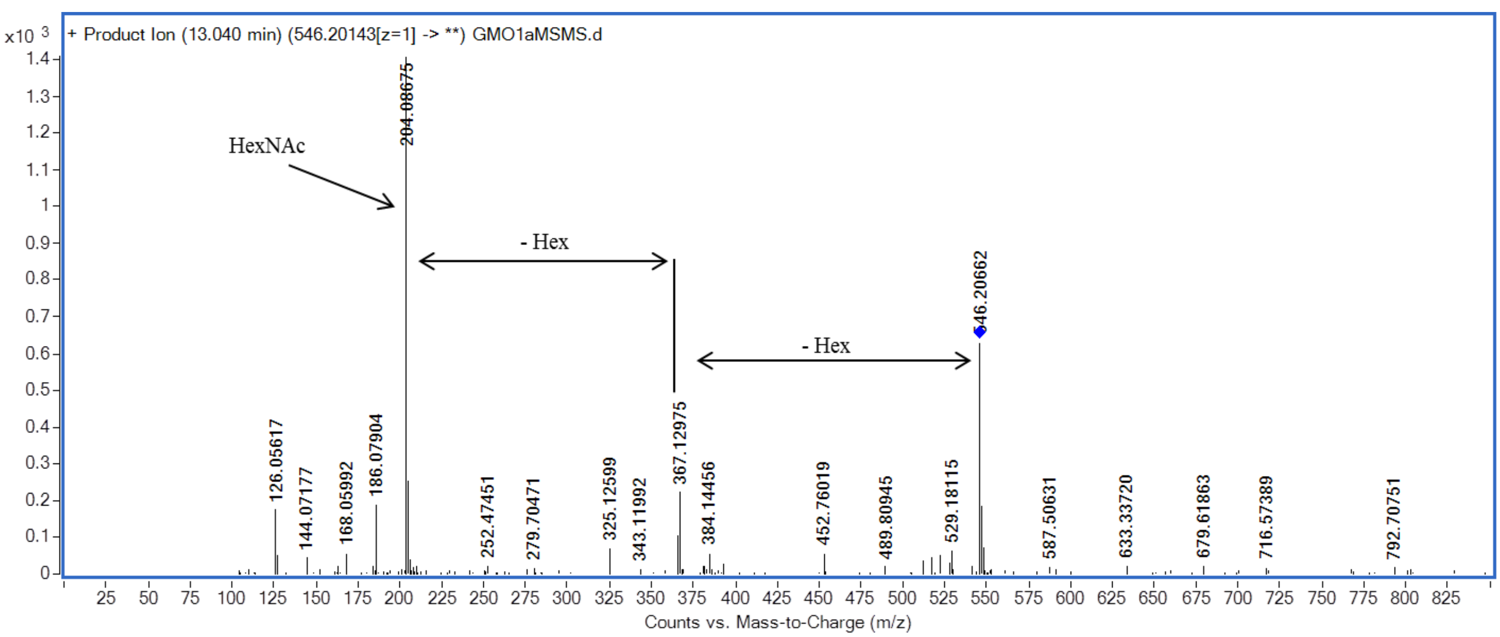
x10 3
+ Product Ion (29.318 min) (925.31423[z=1] -> **) GMO1aMSMS.d
- Neu5Ac
- Neu5Ac
- Hexose
- Hexose
109.32442
141.77177
166.75555
197.04911
227.29855
274.09108
292.10395
308.09679
325.11127
343.11620
384.14721
454.15817
472.43688
498.45120
550.48894
583.19952
616.21191
634.21925
646.22242
691.22879
745.24079
822.38599
885.07438
907.32059
Counts vs. Mass-to-Charge (m/z)

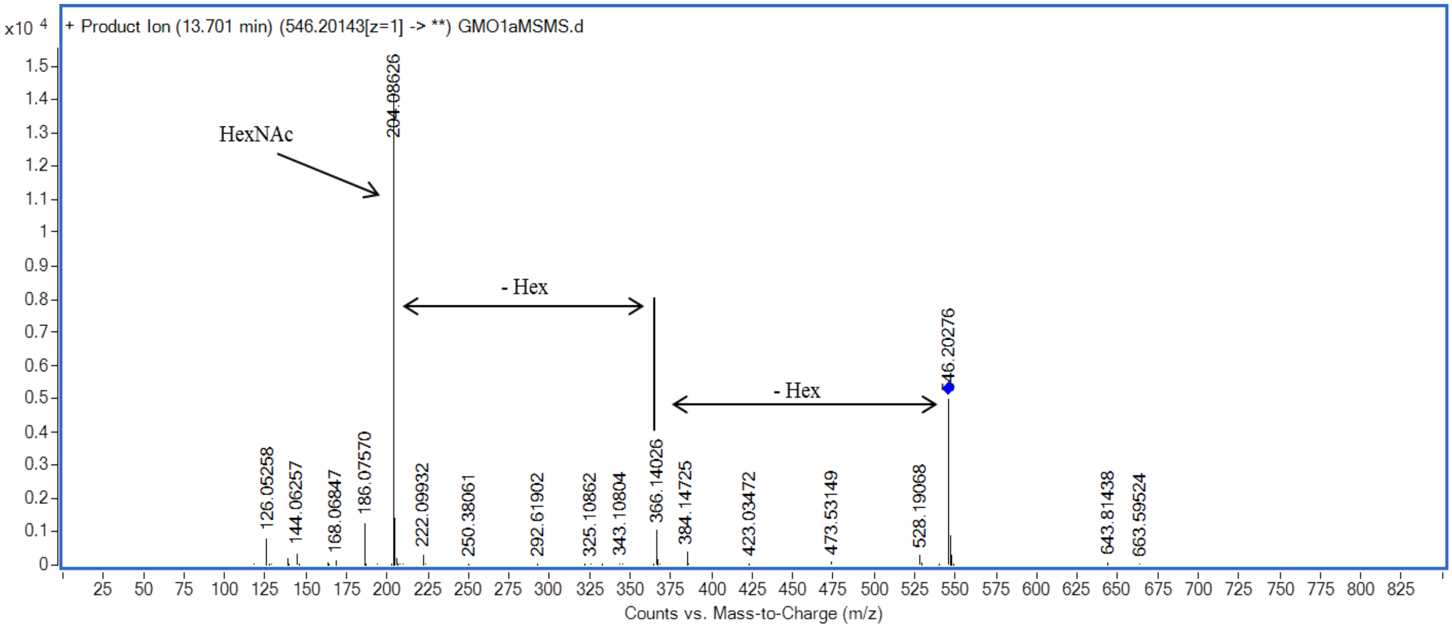
h) 2_0_1_0_0

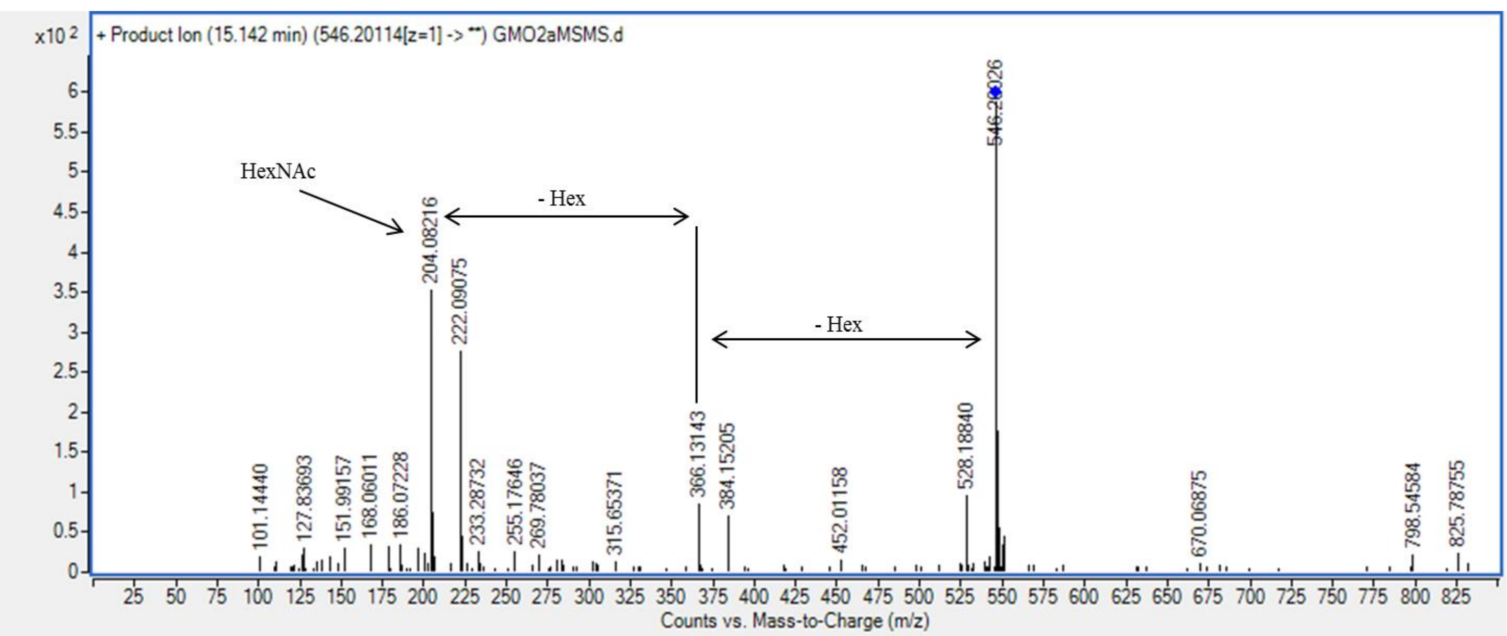

x10 3
+ Product Ion (12.547 min) (489.17950[z=1] -> **) GMO5aMSMS.d
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
163.05898
Hexose
- Hex
325.11535
- Fucose
273.09557
489.17701
398.50159
564.10066
25 50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600
Counts vs. Mass-to-Charge (m/z)

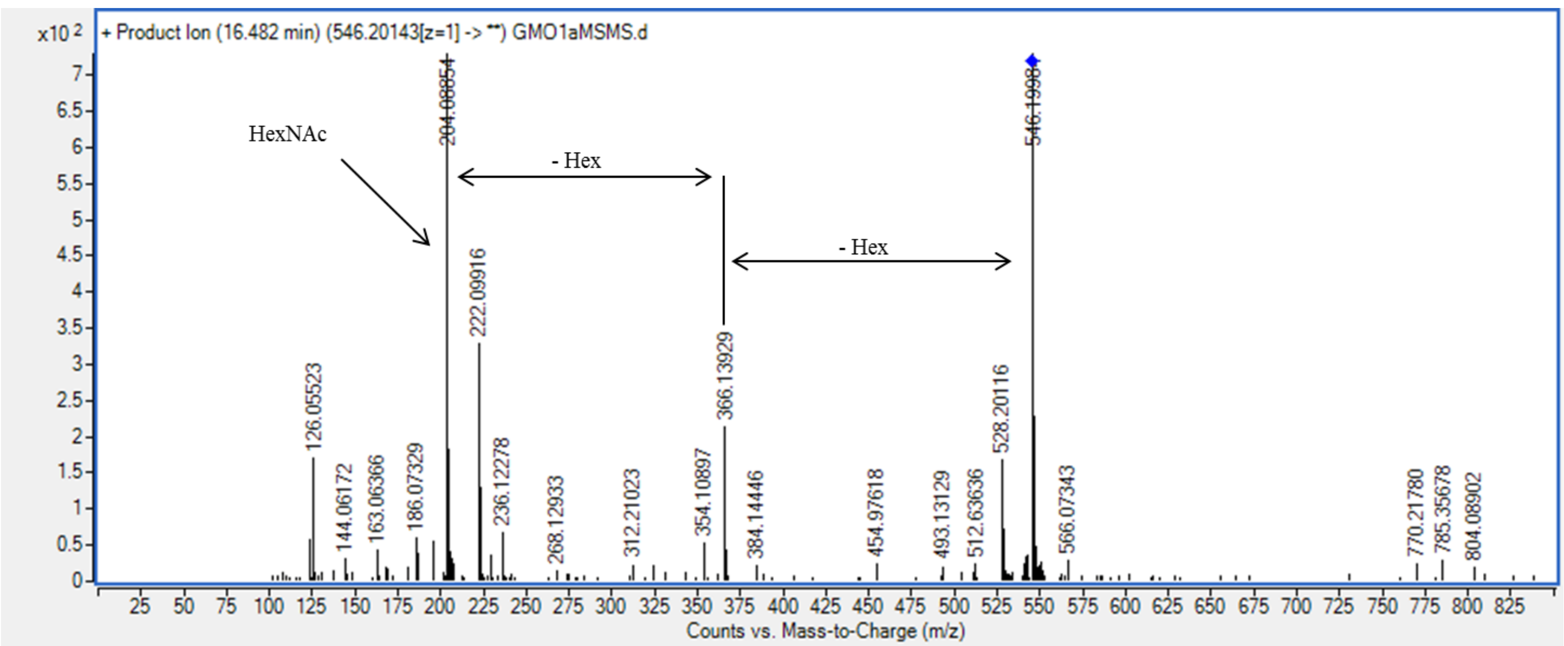
i) 2_1_0_0_0


+ Product Ion (12.396 min) (546.20143[z=1] -> **) GMO1aMSMS.d
x10 3
HexNAc
204.08511
- Hex
- Hex
546.20113
384.14430
112.46987
138.05621
168.06376
186.07176
348.09870
366.13764
452.25440
492.18019
512.43997
528.17899
565.50913
612.99358
645.86125
729.02738
743.29318
782.76570
Counts vs. Mass-to-Charge (m/z)

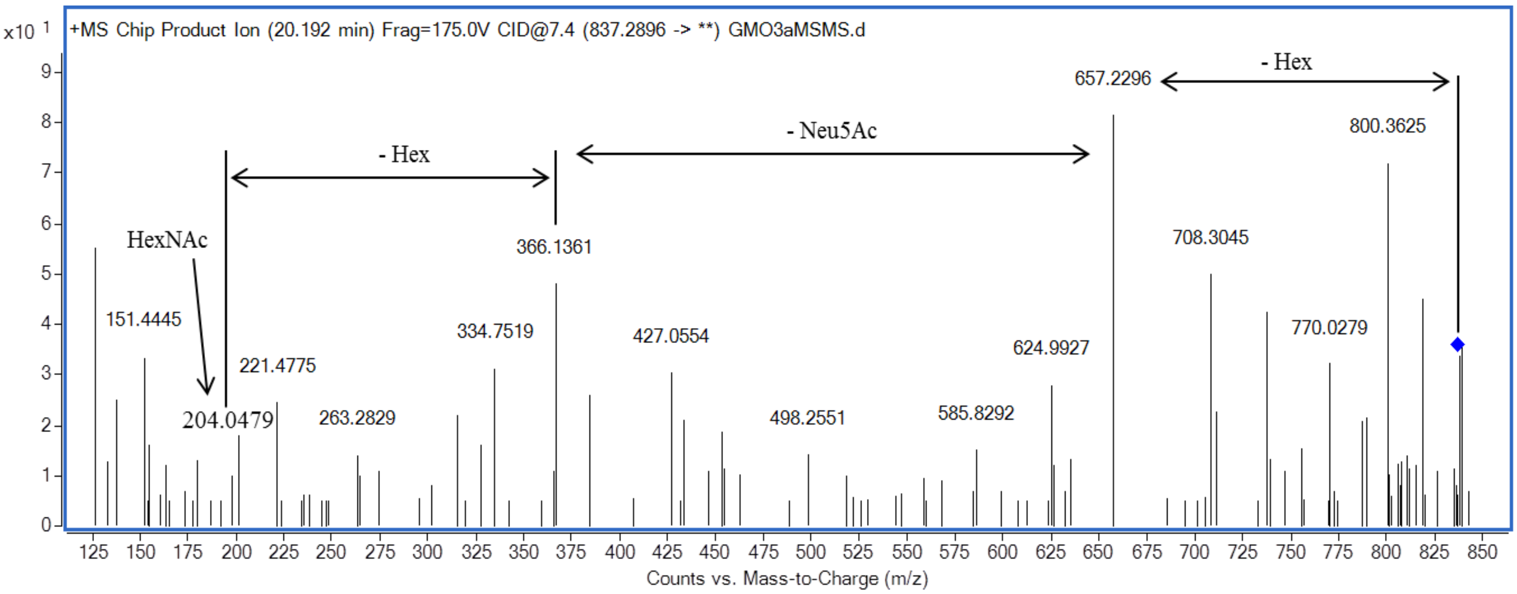

+ Product Ion (13.040 min) (546.20143[z=1] -> **) GMO1aMSMS.d
x10 3
HexNAc
204.08675
- Hex
- Hex
546.20662
126.05617
144.07177
168.05992
186.07904
252.47451
279.70471
325.12599
343.11992
367.12975
384.14456
452.76019
489.80945
529.18115
587.50631
633.33720
679.61863
716.57389
792.70751
Counts vs. Mass-to-Charge (m/z)


x10 4
+ Product Ion (13.701 min) (546.20143[z=1] -> **) GMO1aMSMS.d
HexNAc
- Hex
- Hex
204.08626
546.20276
366.14026
126.05258
144.06257
168.06847
186.07570
222.09932
250.38061
292.61902
325.10862
343.10804
384.14725
423.03472
473.53149
528.19068
643.81438
663.59524
Counts vs. Mass-to-Charge (m/z)


x10 2
+ Product Ion (15.142 min) (546.20114[z=1] -> **) GMO2aMSMS.d
HexNAc
- Hex
- Hex
546.20026
204.08216
222.09075
101.14440
127.83693
151.99157
168.06011
186.07228
233.28732
255.17646
269.78037
315.65371
366.13143
384.15205
452.01158
528.18840
670.06875
798.54584
825.78755
Counts vs. Mass-to-Charge (m/z)

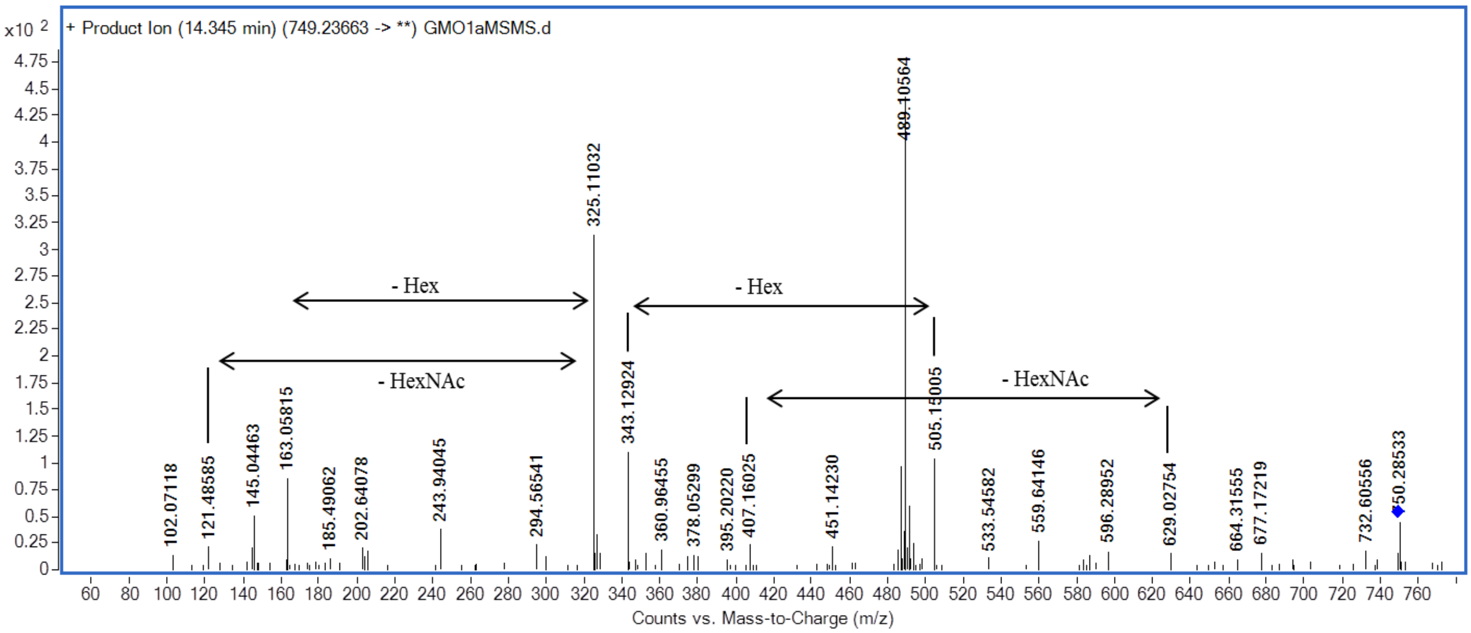

x10 2
+ Product Ion (16.482 min) (546.20143[z=1] -> **) GMO1aMSMS.d
HexNAc
- Hex
- Hex
204.08854
546.19981
222.09916
366.13929
126.05523
144.06172
163.06366
186.07329
236.12278
268.12933
312.21023
354.10897
384.14446
454.97618
493.13129
512.63636
528.20116
566.07343
770.21780
785.35678
804.08902
Counts vs. Mass-to-Charge (m/z)

j) 2_1_0_1_0



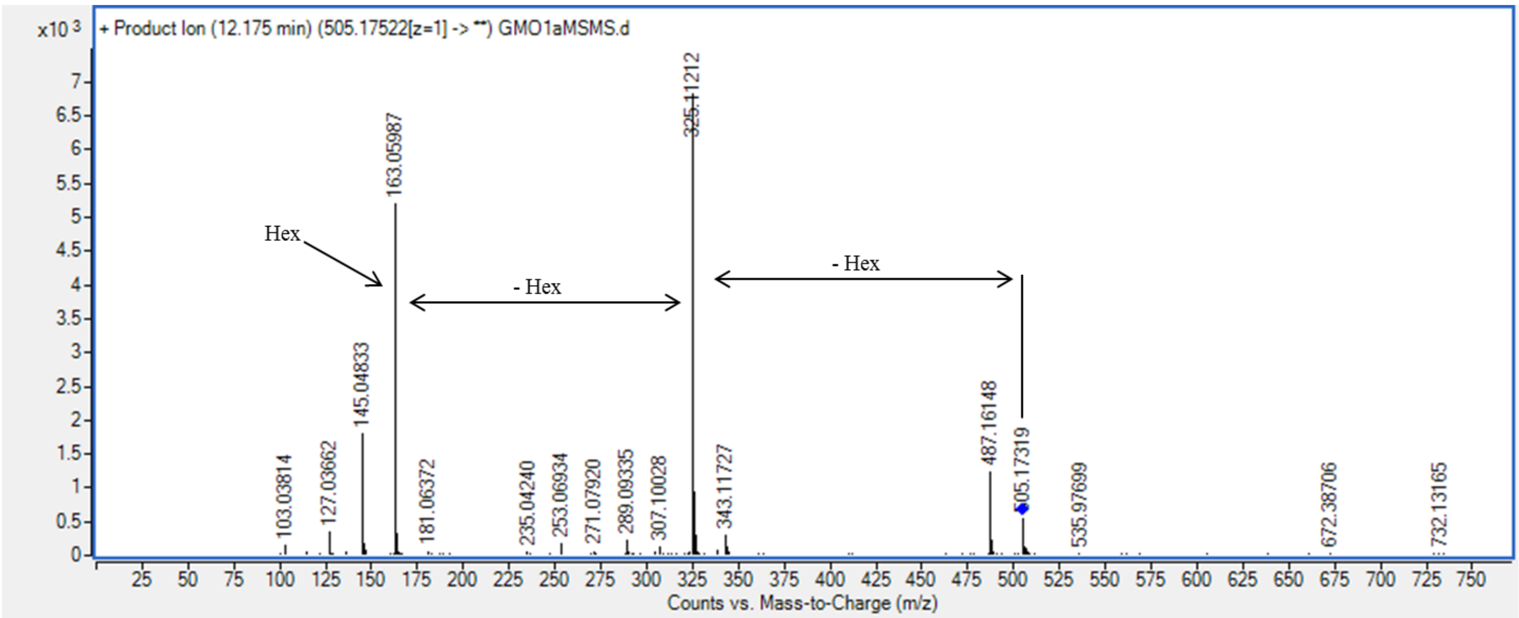
x10 1
+MS Chip Product Ion (20.192 min) Frag=175.0V CID@7.4 (837.2896 -> **) GMO3aMSMS.d
- Hex
657.2296
800.3625
- Neu5Ac
- Hex
HexNAc
366.1361
708.3045
151.4445
334.7519
427.0554
770.0279
624.9927
221.4775
204.0479
263.2829
498.2551
585.8292
Counts vs. Mass-to-Charge (m/z)

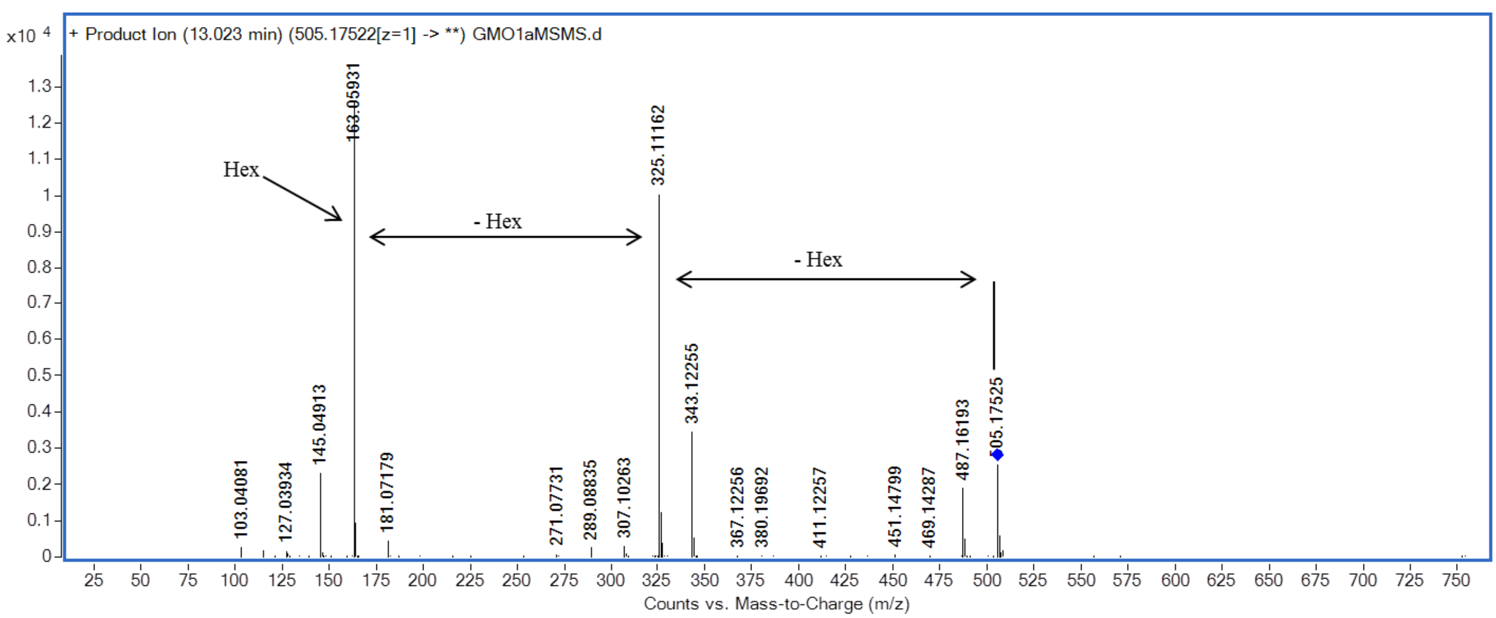
k) 2_2_0_0_0

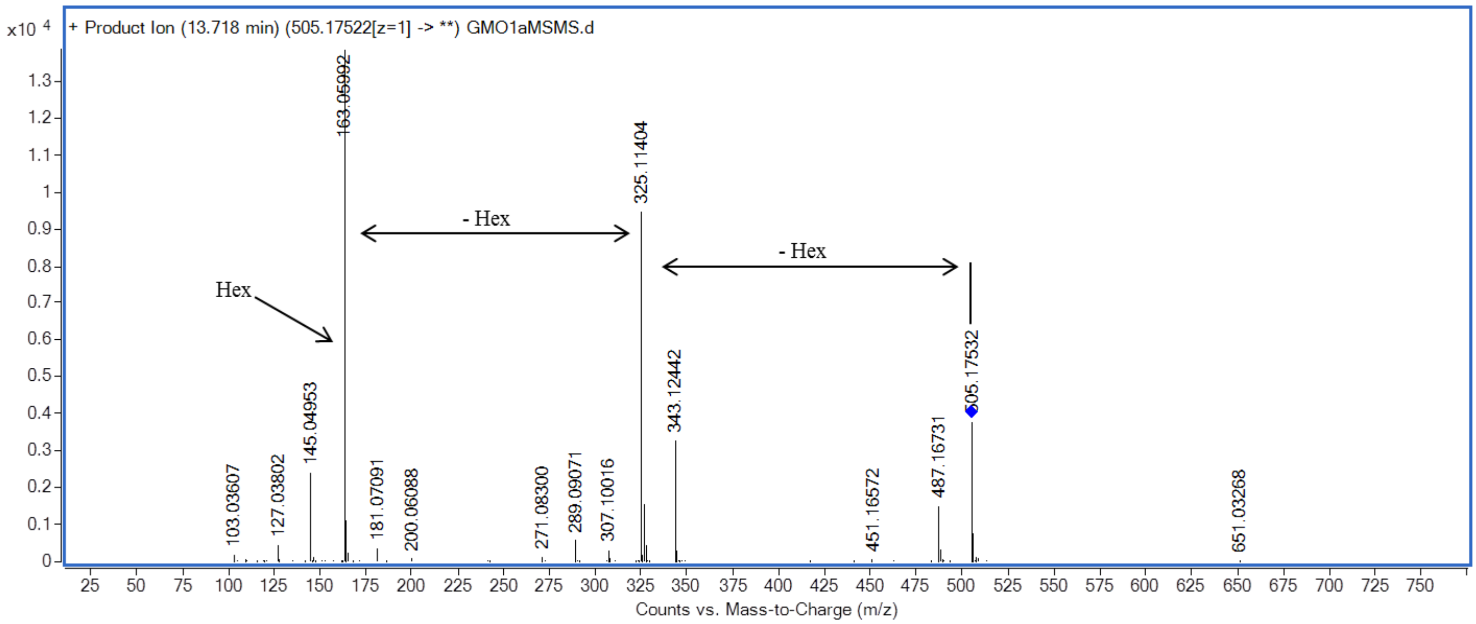


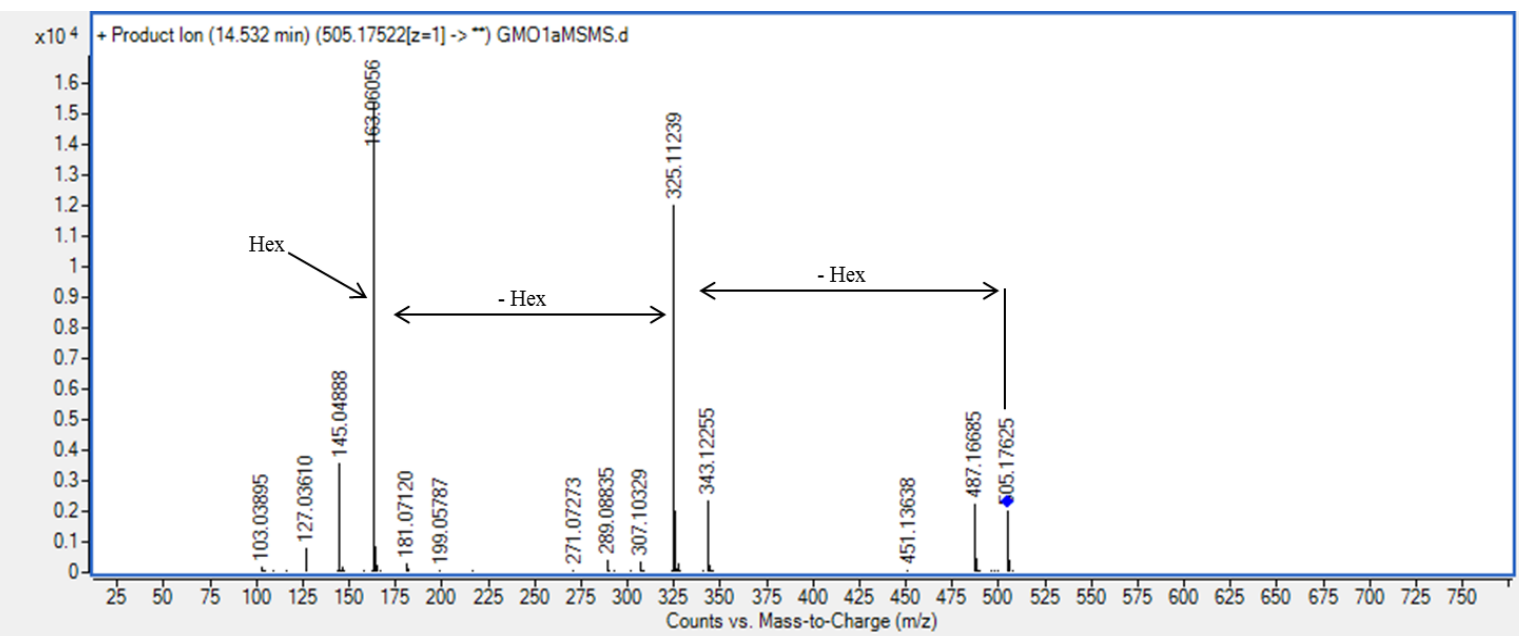
x10 2
+ Product Ion (14.345 min) (749.23663 -> **) GMO1aMSMS.d
- Hex
- Hex
- HexNAc
- HexNAc
102.07118
121.48585
145.04463
163.05815
185.49062
202.64078
243.94045
294.56541
325.11032
343.12924
360.96455
378.05299
395.20220
407.16025
451.14230
489.10564
505.15005
533.54582
559.64146
596.28952
629.02754
664.31555
677.17219
732.60556
750.28533
Counts vs. Mass-to-Charge (m/z)

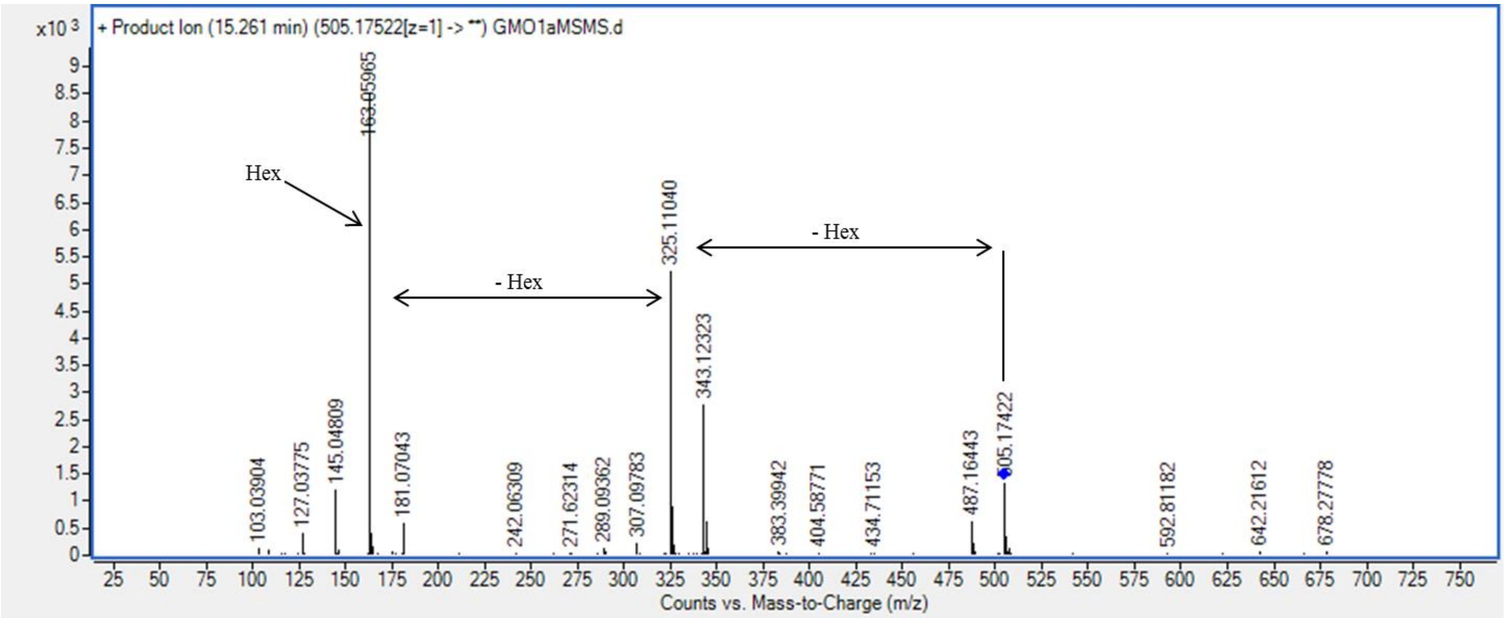
l) 3_0_0_0_0

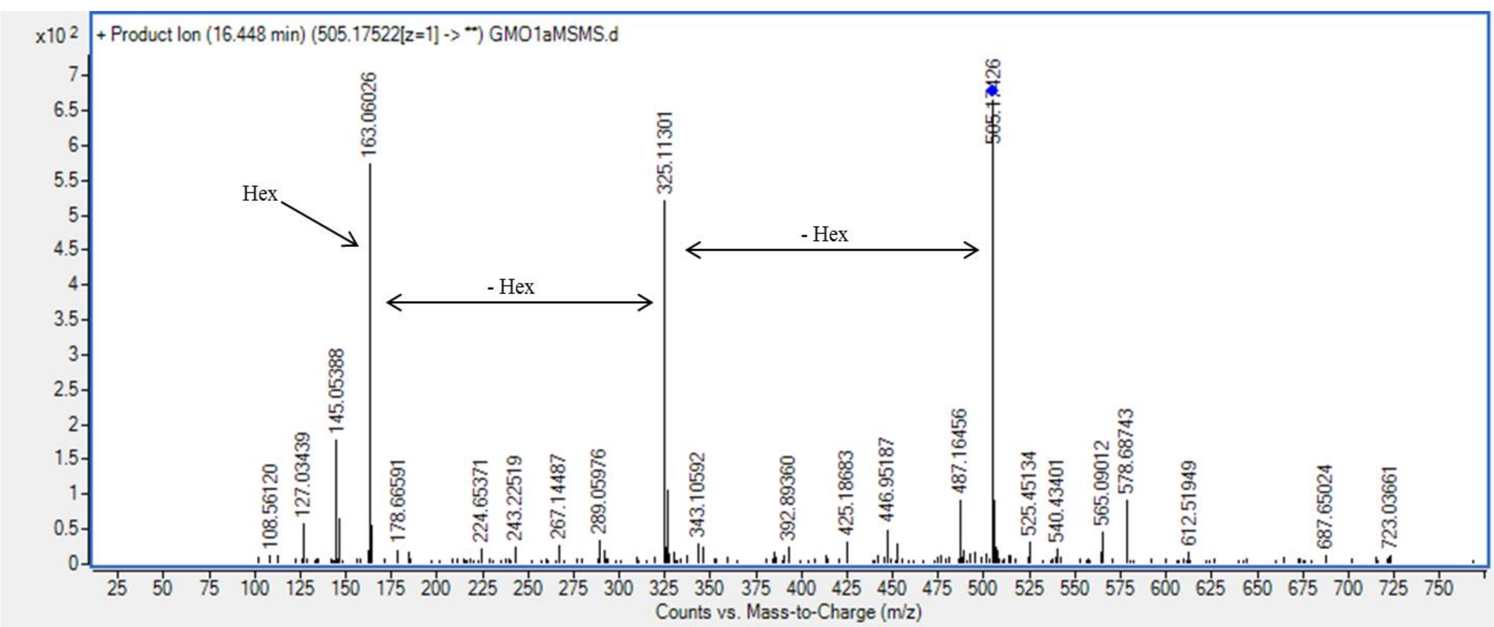

x10 3
+ Product Ion (12.175 min) (505.17522[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
103.03814
127.03662
145.04833
163.05987
181.06372
235.04240
253.06934
271.07920
289.09335
307.10028
325.11212
343.11727
487.16148
505.17319
535.97699
672.38706
732.13165
Counts vs. Mass-to-Charge (m/z)

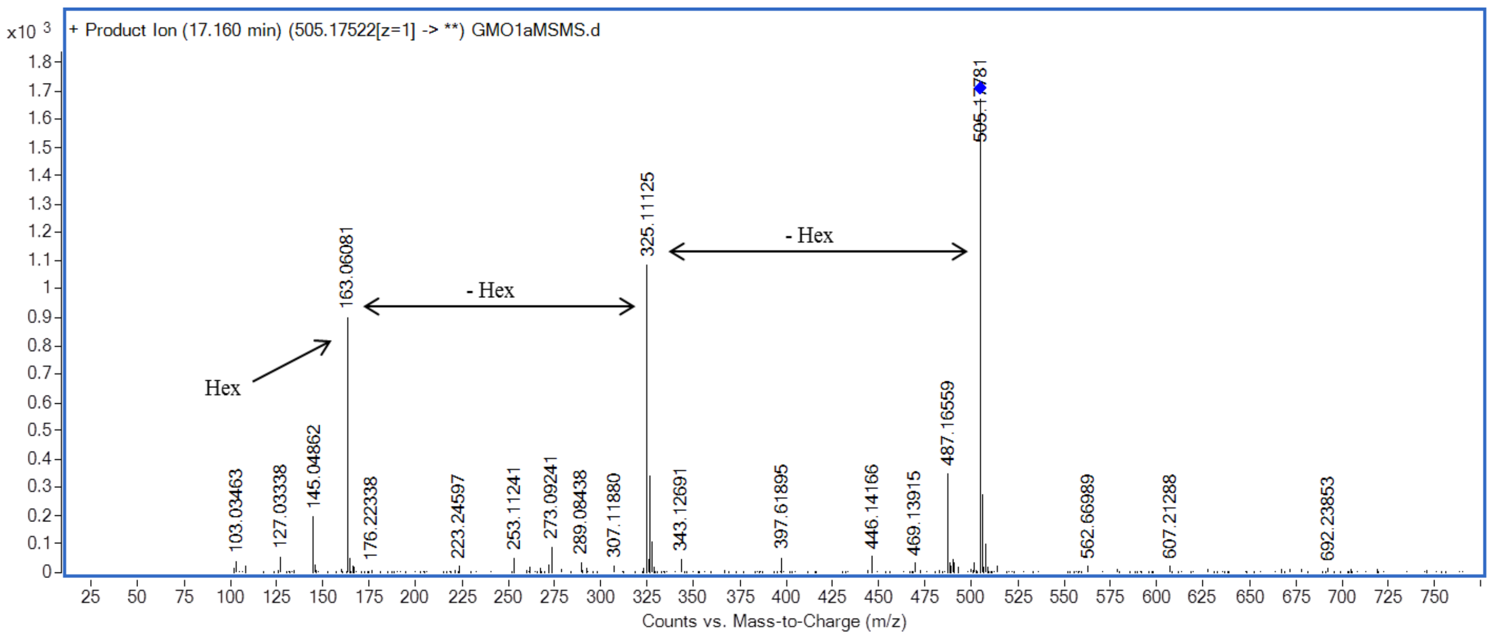

x10 4
+ Product Ion (13.023 min) (505.17522[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
103.04081
127.03934
145.04913
163.05931
181.07179
271.07731
289.08835
307.10263
325.11162
343.12255
367.12256
380.19692
411.12257
451.14799
469.14287
487.16193
505.17525
Counts vs. Mass-to-Charge (m/z)


x10 4
+ Product Ion (13.718 min) (505.17522[z=1] -> **) GMO1aMSMS.d
163.05992
325.11404
- Hex
- Hex
Hex
343.12442
505.17532
145.04953
487.16731
103.03607
127.03802
181.07091
200.06088
271.08300
289.09071
307.10016
451.16572
651.03268
Counts vs. Mass-to-Charge (m/z)


x10 4
+ Product Ion (14.532 min) (505.17522[z=1] -> **) GMO1aMSMS.d
163.06056
325.11239
Hex
- Hex
- Hex
145.04888
343.12255
487.16685
505.17625
103.03895
127.03610
181.07120
199.05787
271.07273
289.08835
307.10329
451.13638
Counts vs. Mass-to-Charge (m/z)

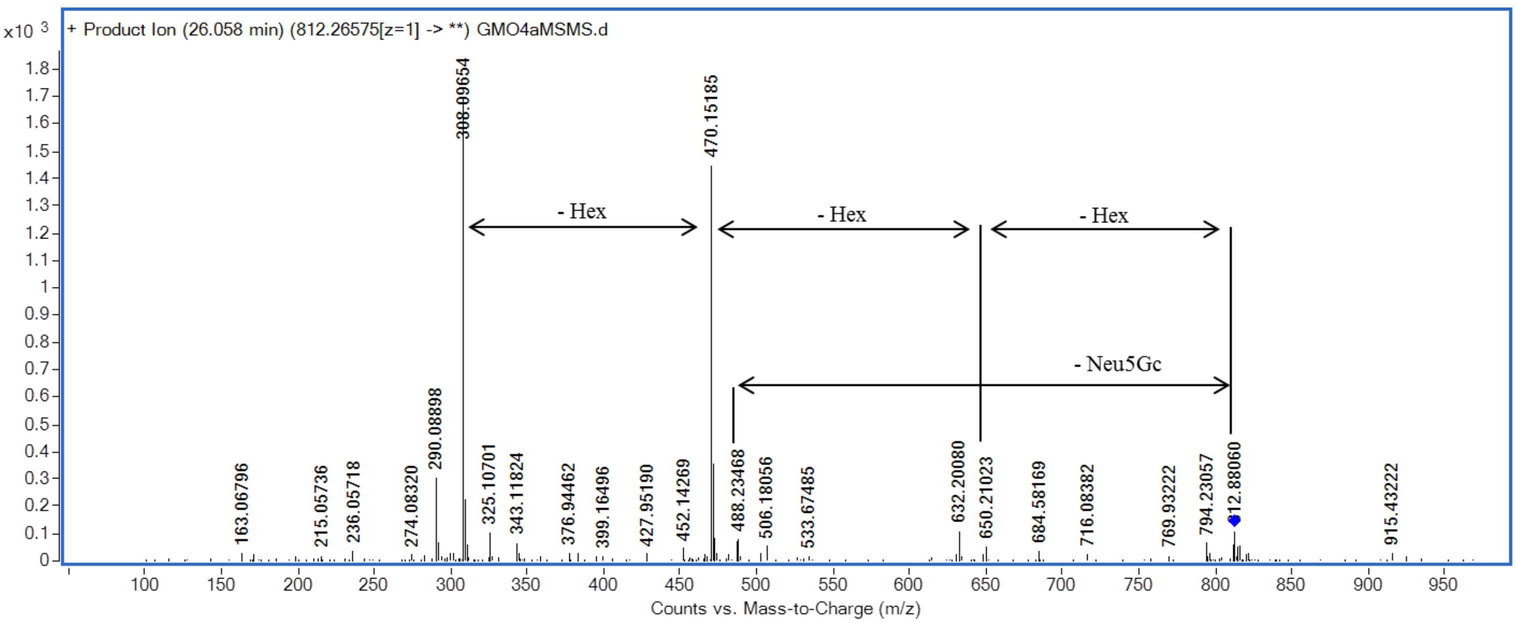

x10 3
+ Product Ion (15.261 min) (505.17522[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
163.05965
325.11040
343.12323
103.03904
127.03775
145.04809
181.07043
242.06309
271.62314
289.09362
307.09783
383.39942
404.58771
434.71153
487.16443
505.17422
592.81182
642.21612
678.27778
Counts vs. Mass-to-Charge (m/z)


x10 2
+ Product Ion (16.448 min) (505.17522[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
163.06026
325.11301
108.56120
127.03439
145.05388
178.66591
224.65371
243.22519
267.14487
289.05976
343.10592
392.89360
425.18683
446.95187
487.16456
525.45134
540.43401
565.09012
578.68743
612.51949
687.65024
723.03661
Counts vs. Mass-to-Charge (m/z)

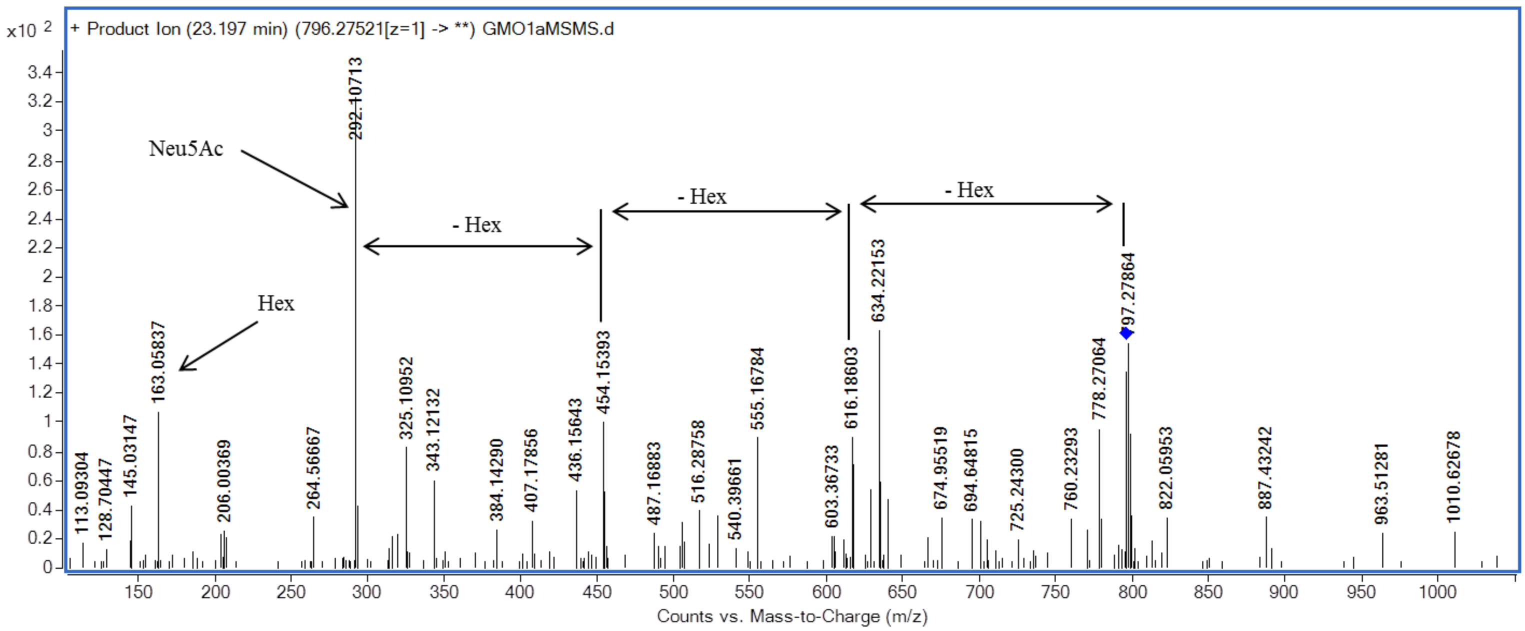


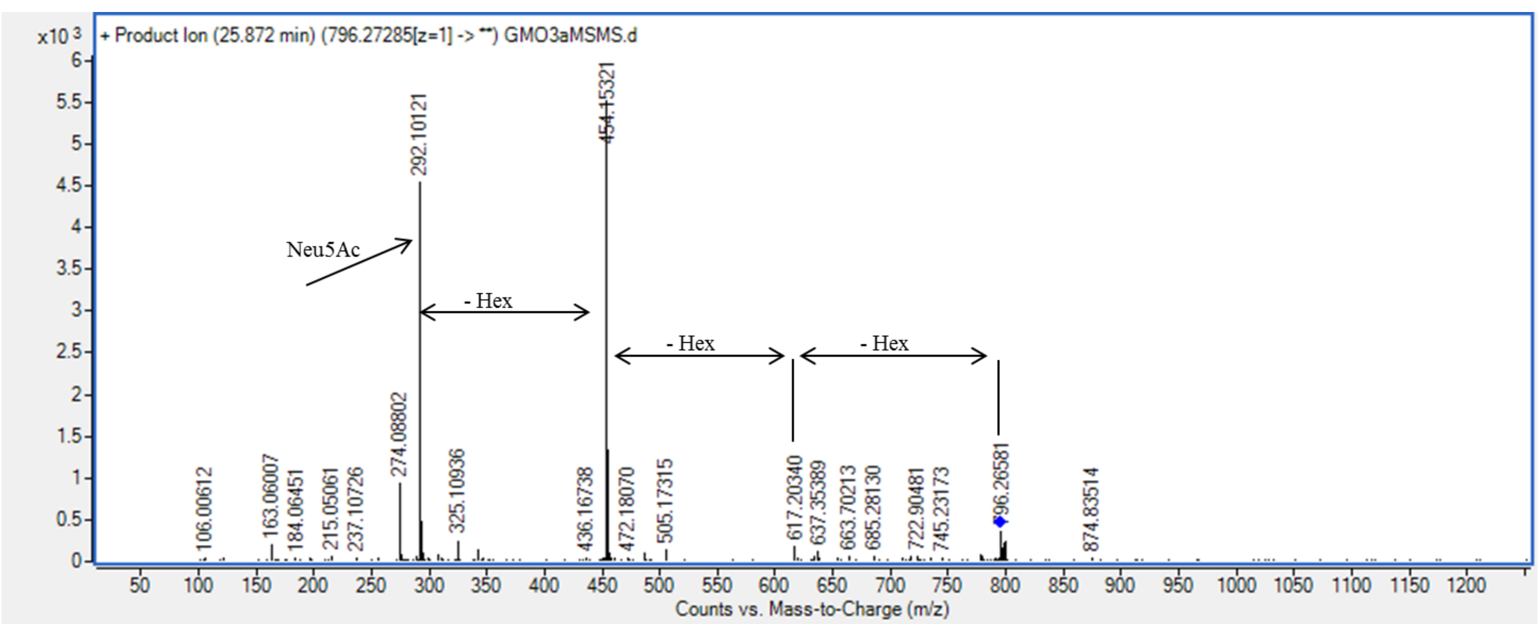
x10 3
+ Product Ion (17.160 min) (505.17522[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
103.03463
127.03338
145.04862
163.06081
176.22338
223.24597
253.11241
273.09241
289.08438
307.11880
325.11125
343.12691
397.61895
446.14166
469.13915
487.16559
505.17781
562.66989
607.21288
692.23853
Counts vs. Mass-to-Charge (m/z)

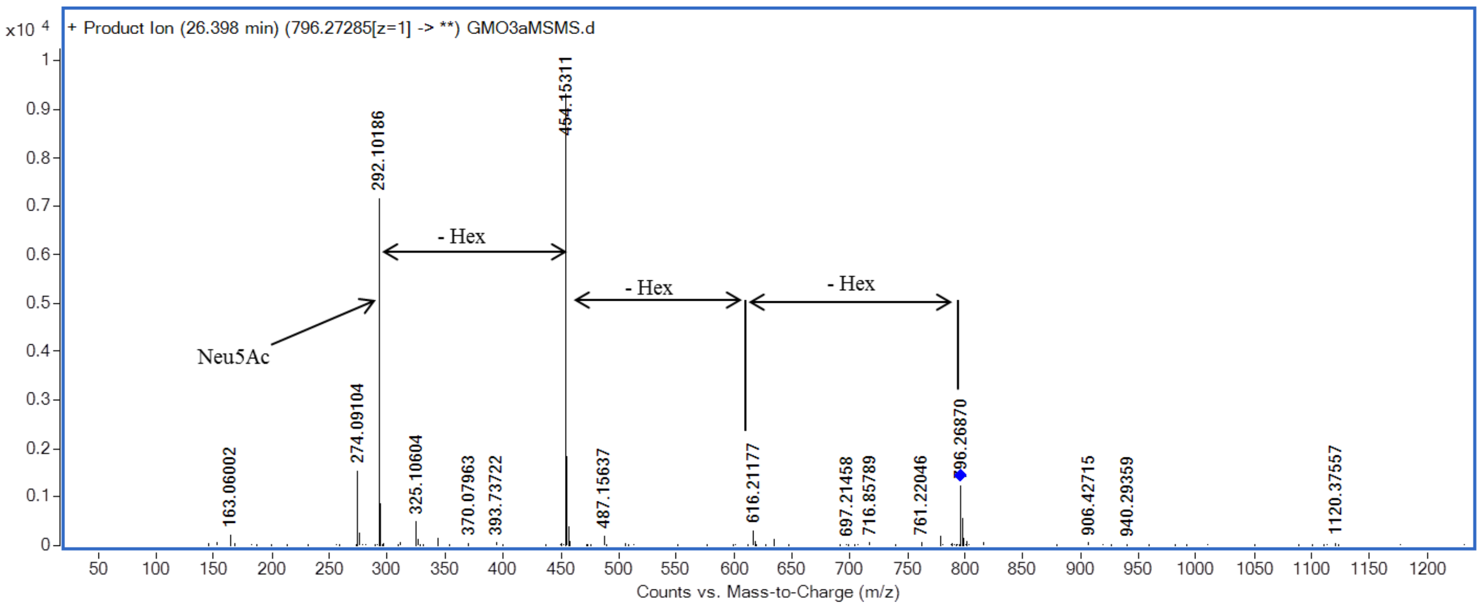
m) 3_0_0_0_1


x10 3
+ Product Ion (26.058 min) (812.26575[z=1] -> **) GMO4aMSMS.d
308.09654
470.15185
- Hex
- Hex
- Hex
- Neu5Gc
163.06796
215.05736
236.05718
274.08320
290.08898
325.10701
343.11824
376.94462
399.16496
427.95190
452.14269
488.23468
506.18056
533.67485
632.20080
650.21023
684.58169
716.08382
769.93222
794.23057
812.88060
915.43222
Counts vs. Mass-to-Charge (m/z)

n) 3_0_0_1_0

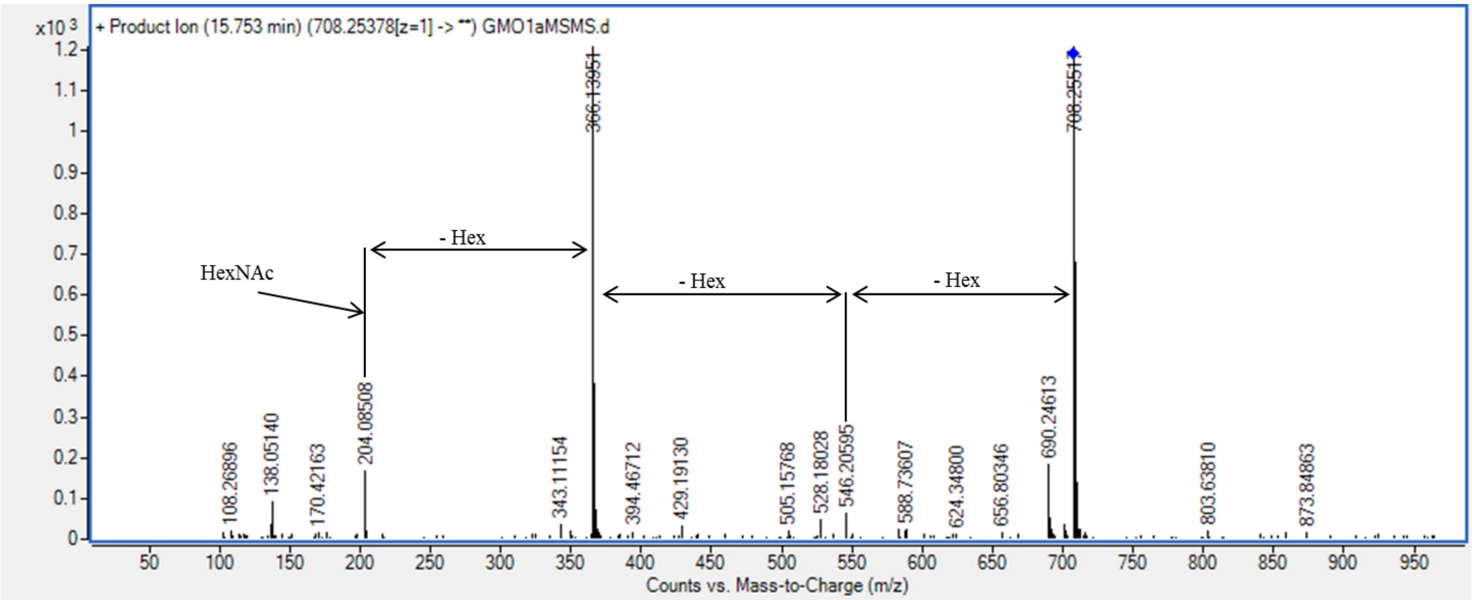

x10 2
+ Product Ion (23.197 min) (796.27521[z=1] -> **) GMO1aMSMS.d
Neu5Ac
Hex
- Hex
- Hex
- Hex
292.10713
163.05837
454.15393
616.18603
634.22153
797.27864
778.27064
Counts vs. Mass-to-Charge (m/z)


x10 3
+ Product Ion (25.872 min) (796.27285[z=1] -> **) GMO3aMSMS.d
Neu5Ac
- Hex
- Hex
- Hex
292.10121
454.15321
274.08802
617.20340
796.26581
Counts vs. Mass-to-Charge (m/z)

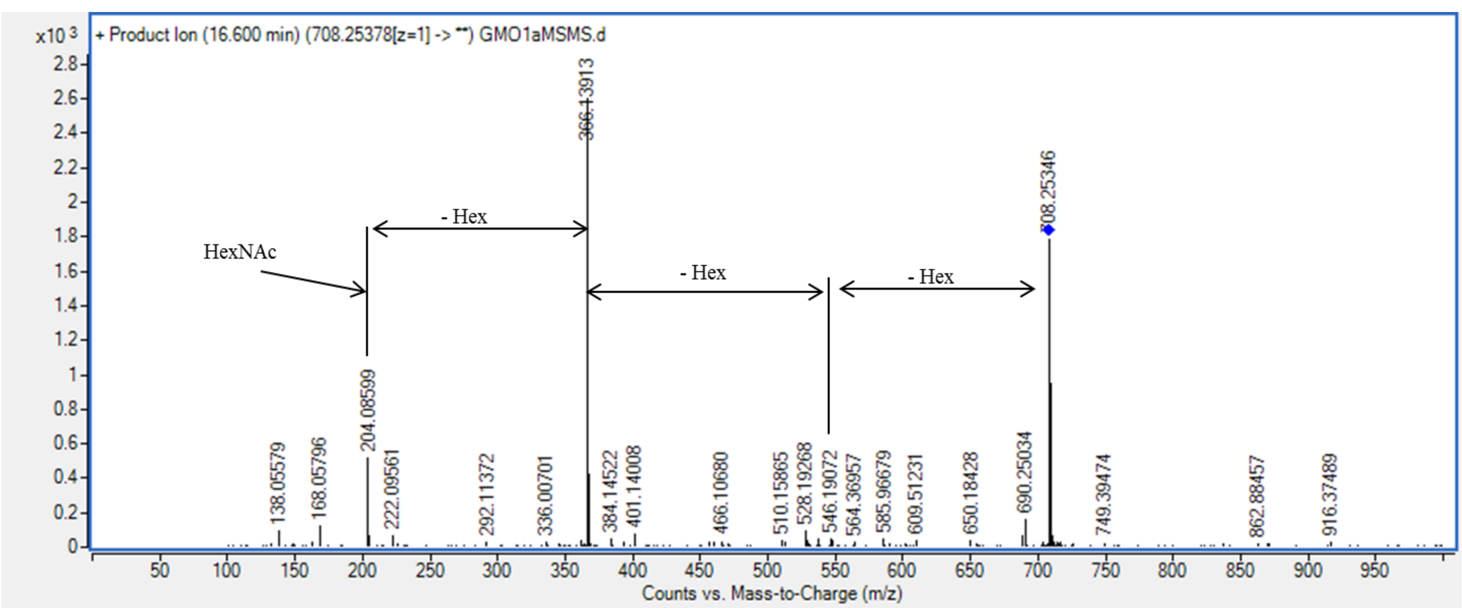


x10 4
+ Product Ion (26.398 min) (796.27285[z=1] -> **) GMO3aMSMS.d
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
454.15311
292.10186
- Hex
- Hex
- Hex
Neu5Ac
274.09104
796.26870
163.06002
325.10604
370.07963
393.73722
487.15637
616.21177
697.21458
716.85789
761.22046
906.42715
940.29359
1120.37557
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
1150
1200
Counts vs. Mass-to-Charge (m/z)

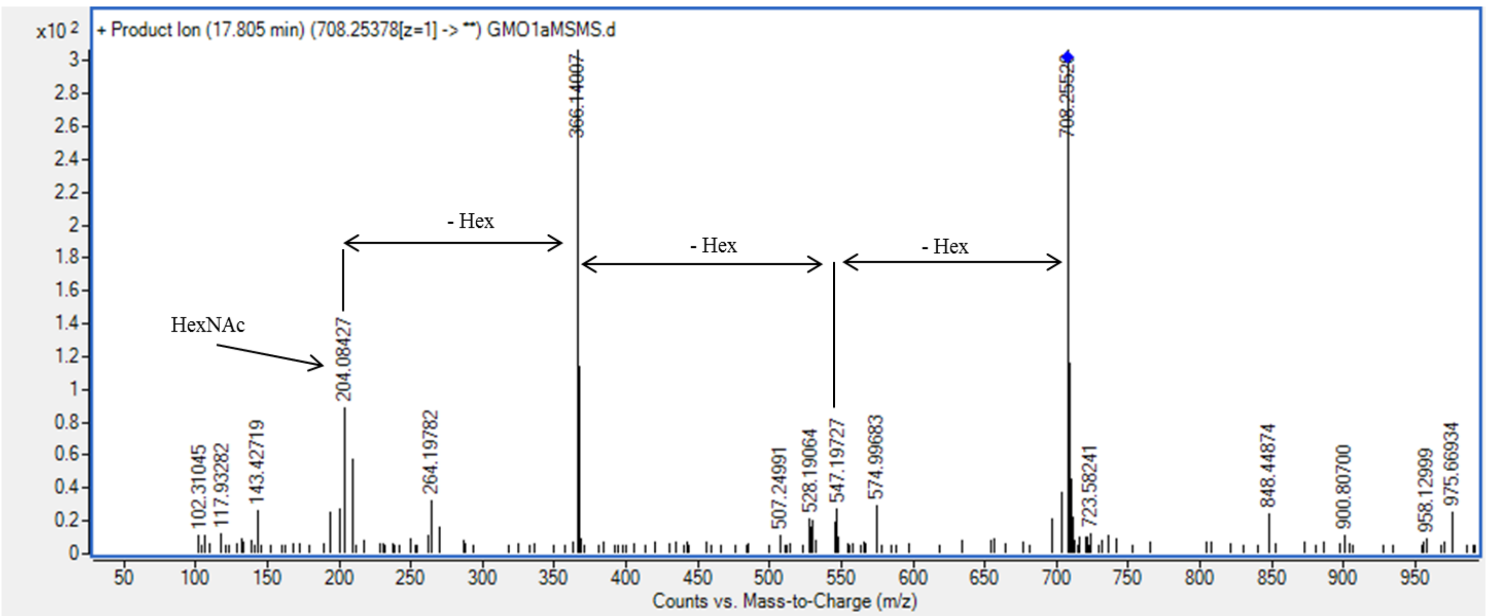
o) 3_1_0_0_0



x10 3
+ Product Ion (15.753 min) (708.25378[z=1] -> **) GMO1aMSMS.d
HexNAc
- Hex
- Hex
- Hex
366.13951
708.25514
108.26896
138.05140
170.42163
204.08508
343.11154
394.46712
429.19130
505.15768
528.18028
546.20595
588.73607
624.34800
656.80346
690.24613
803.63810
873.84863
Counts vs. Mass-to-Charge (m/z)

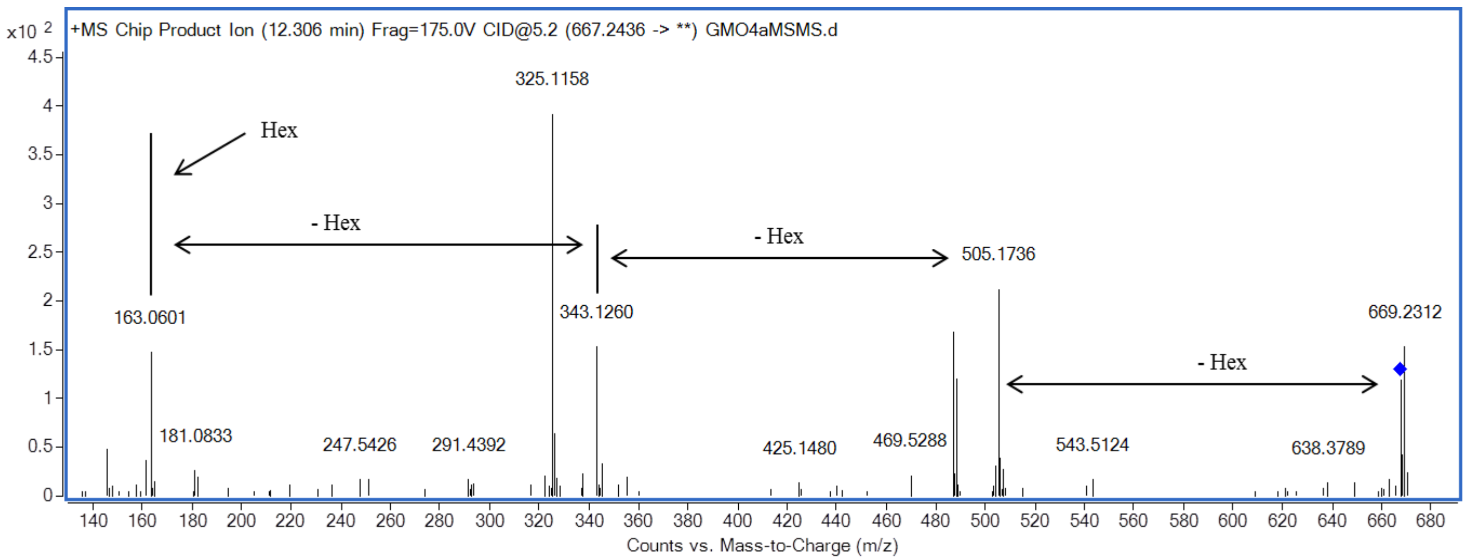


x10 3
+ Product Ion (16.600 min) (708.25378[z=1] -> **) GMO1aMSMS.d
HexNAc
- Hex
- Hex
- Hex
366.13913
708.25346
138.05579
168.05796
204.08599
222.09561
292.11372
336.00701
384.14522
401.14008
466.10680
510.15865
528.19268
546.19072
564.36957
585.96679
609.51231
650.18428
690.25034
749.39474
862.88457
916.37489
Counts vs. Mass-to-Charge (m/z)

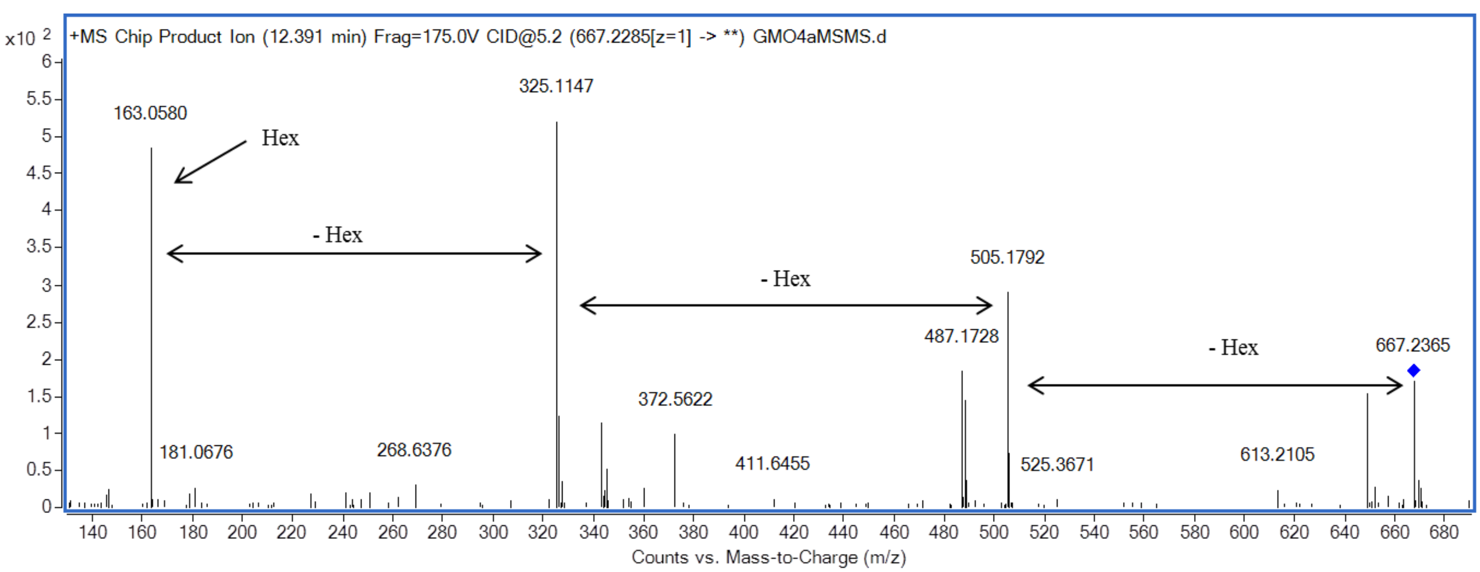


x10 2
+ Product Ion (17.805 min) (708.25378[z=1] -> **) GMO1aMSMS.d
366.14007
708.2552
- Hex
- Hex
- Hex
HexNAc
204.08427
264.19782
102.31045
117.93282
143.42719
507.24991
528.19064
547.19727
574.99683
723.58241
848.44874
900.80700
958.12999
975.66934
Counts vs. Mass-to-Charge (m/z)

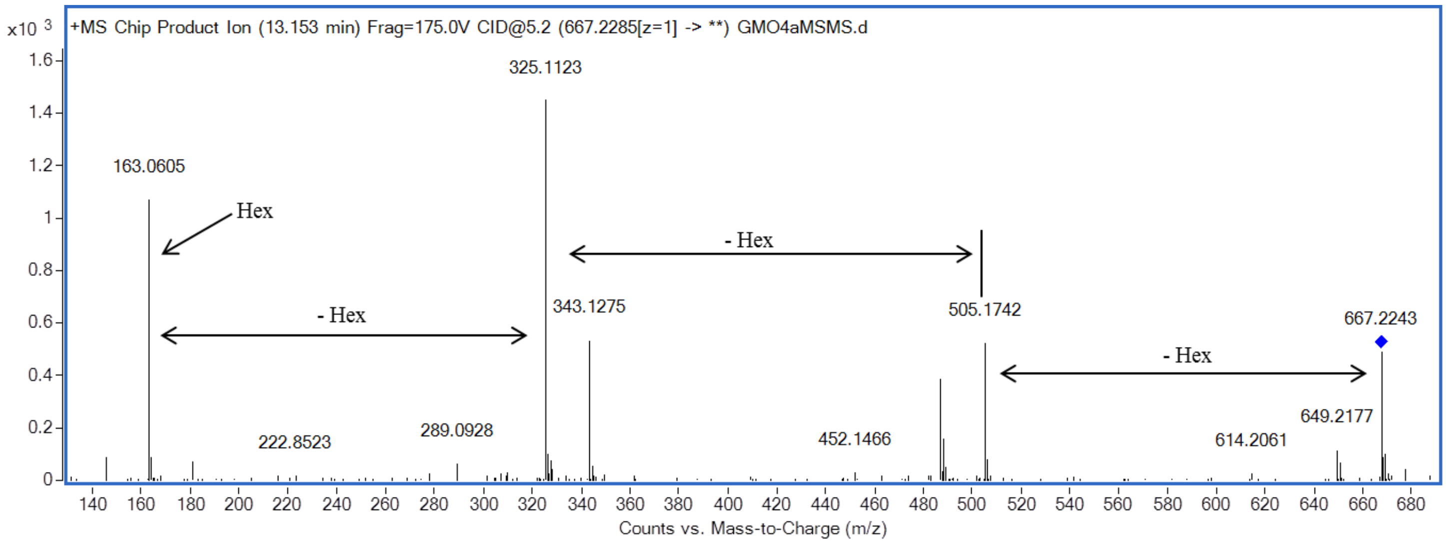
p) 4_0_0_0_0

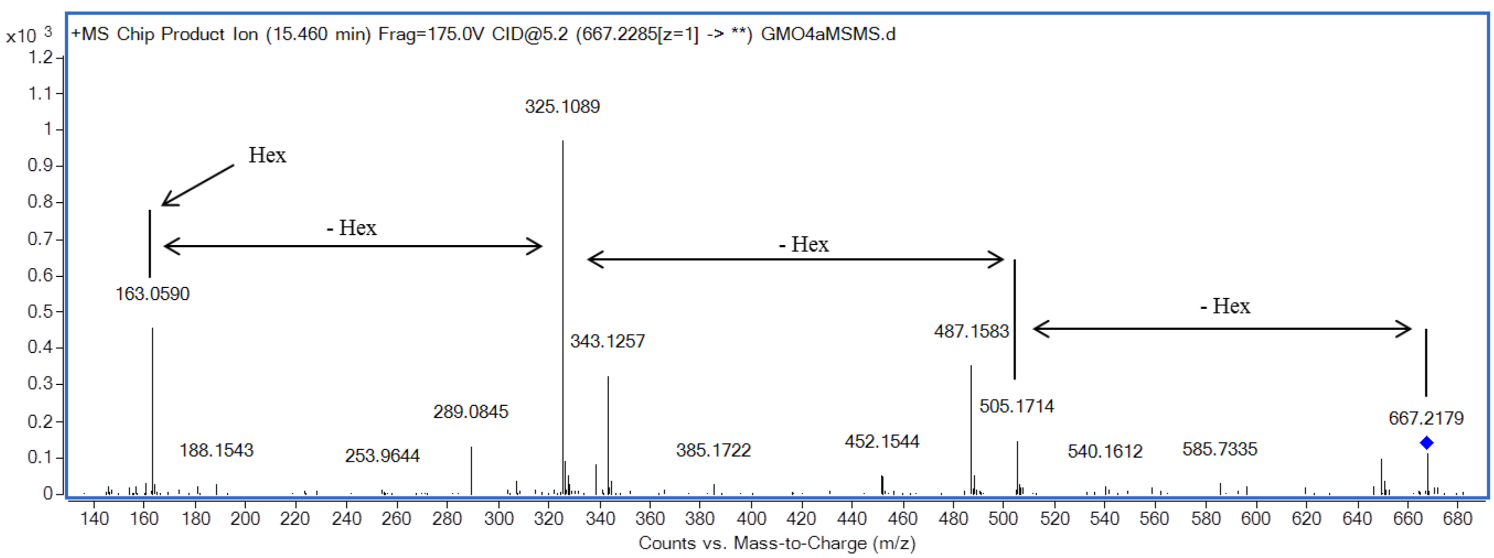


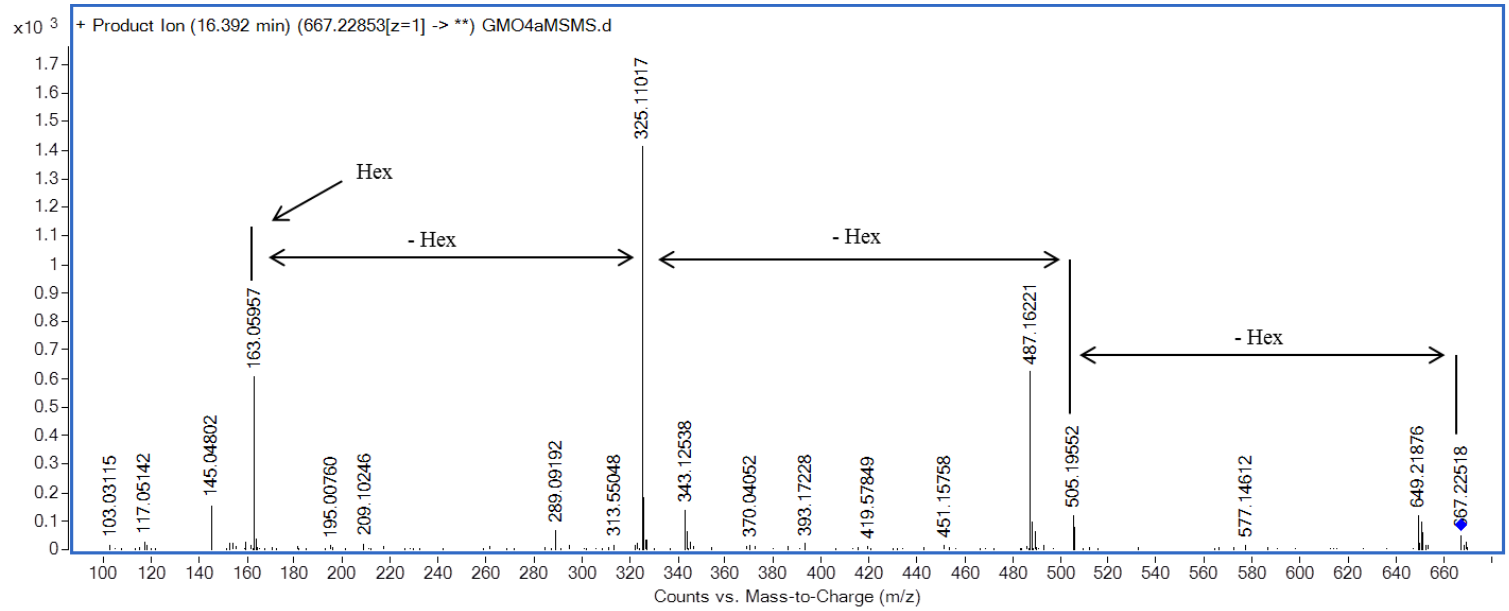
x10 2
+MS Chip Product Ion (12.306 min) Frag=175.0V CID@5.2 (667.2436 -> **) GMO4aMSMS.d
325.1158
Hex
- Hex
- Hex
505.1736
163.0601
343.1260
669.2312
- Hex
181.0833
247.5426
291.4392
425.1480
469.5288
543.5124
638.3789
Counts vs. Mass-to-Charge (m/z)

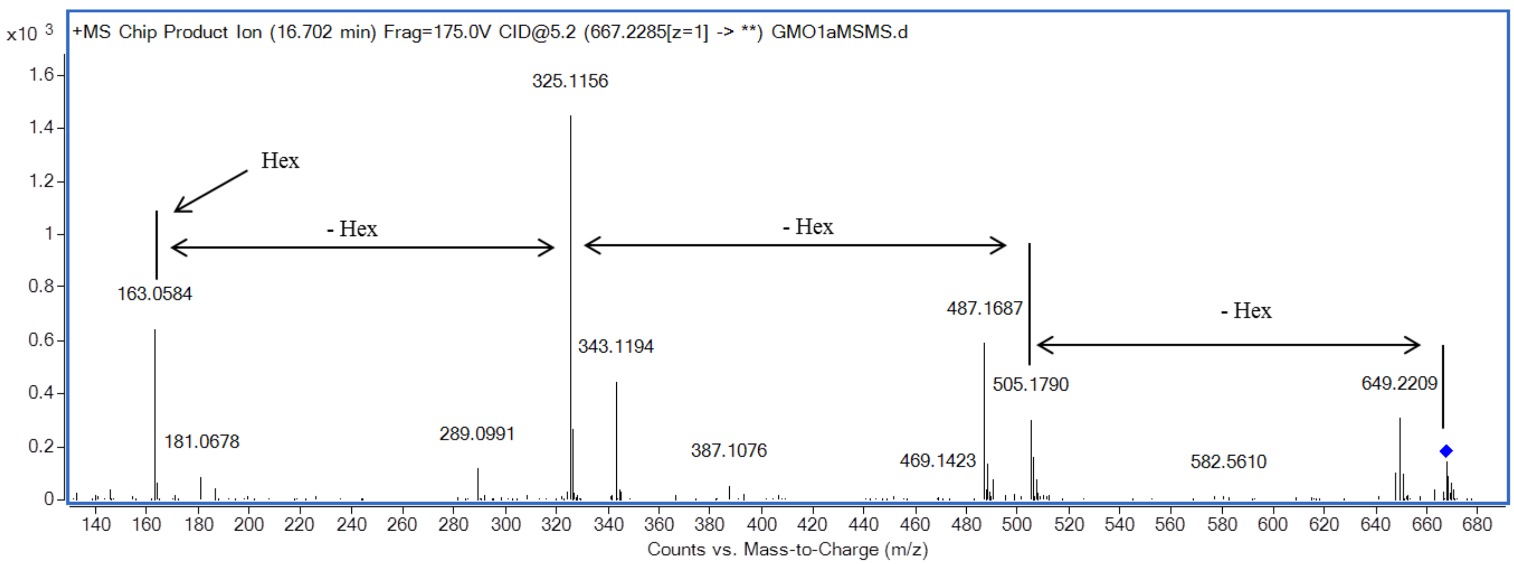


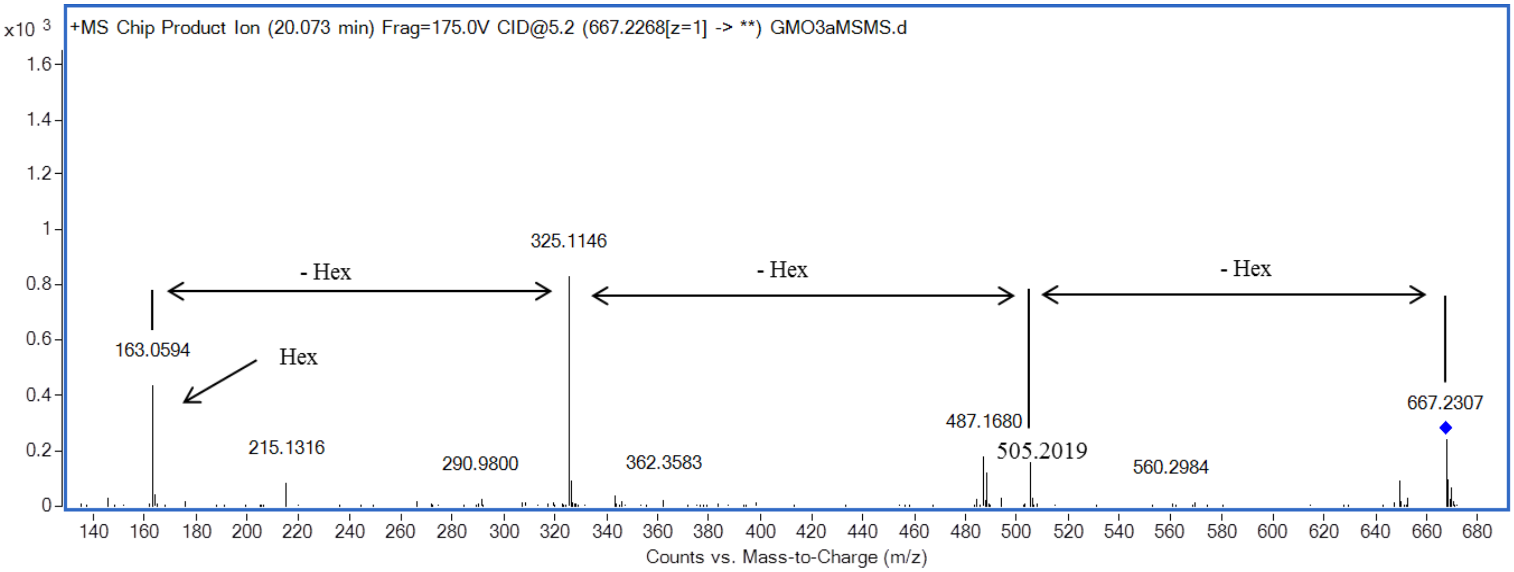
x10 2
+MS Chip Product Ion (12.391 min) Frag=175.0V CID@5.2 (667.2285[z=1] -> **) GMO4aMSMS.d
325.1147
163.0580
Hex
- Hex
505.1792
- Hex
487.1728
- Hex
667.2365
372.5622
181.0676
268.6376
411.6455
525.3671
613.2105
Counts vs. Mass-to-Charge (m/z)

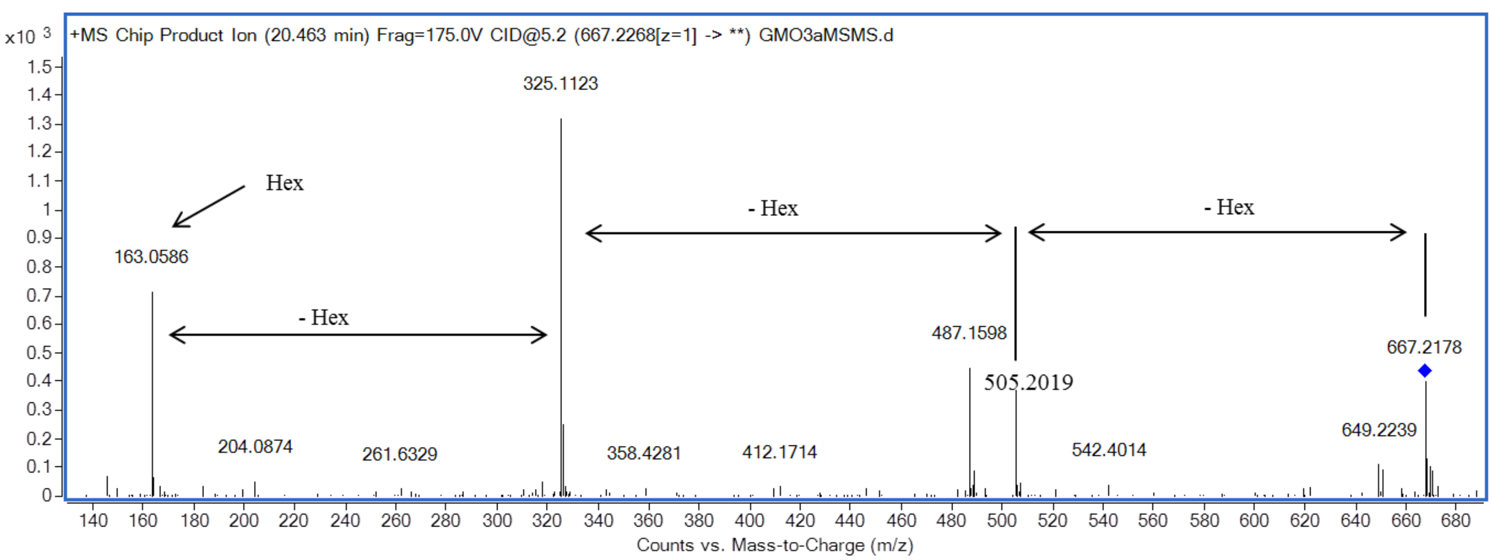

x10 3
+MS Chip Product Ion (13.153 min) Frag=175.0V CID@5.2 (667.2285[z=1] -> **) GMO4aMSMS.d
325.1123
163.0605
Hex
- Hex
- Hex
343.1275
505.1742
667.2243
- Hex
649.2177
614.2061
452.1466
289.0928
222.8523
Counts vs. Mass-to-Charge (m/z)

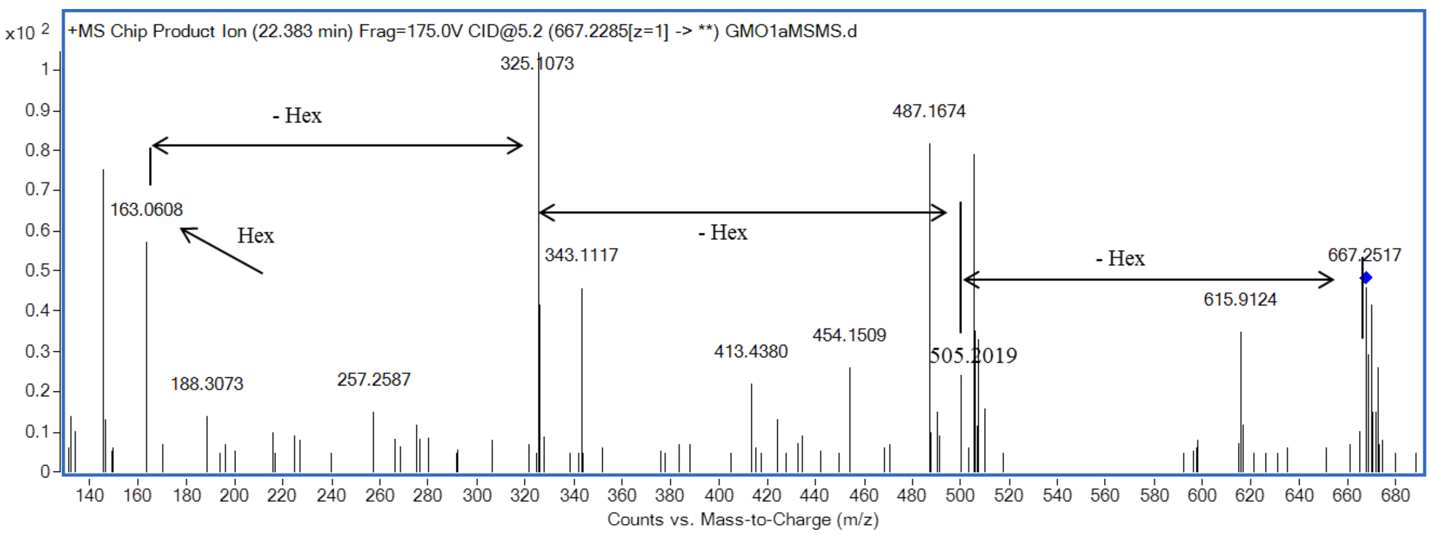

x10 3
+MS Chip Product Ion (15.460 min) Frag=175.0V CID@5.2 (667.2285[z=1] -> **) GMO4aMSMS.d
325.1089
Hex
- Hex
- Hex
163.0590
- Hex
343.1257
487.1583
505.1714
289.0845
667.2179
188.1543
253.9644
385.1722
452.1544
540.1612
585.7335
Counts vs. Mass-to-Charge (m/z)


x10 3
+ Product Ion (16.392 min) (667.22853[z=1] -> **) GMO4aMSMS.d
Hex
- Hex
- Hex
- Hex
103.03115
117.05142
145.04802
163.05957
195.00760
209.10246
289.09192
313.55048
325.11017
343.12538
370.04052
393.17228
419.57849
451.15758
487.16221
505.19552
577.14612
649.21876
667.22518
Counts vs. Mass-to-Charge (m/z)


x10 3
+MS Chip Product Ion (16.702 min) Frag=175.0V CID@5.2 (667.2285[z=1] -> **) GMO1aMSMS.d
Hex
- Hex
- Hex
- Hex
325.1156
163.0584
181.0678
289.0991
343.1194
387.1076
469.1423
487.1687
505.1790
582.5610
649.2209
Counts vs. Mass-to-Charge (m/z)


x10 3
+MS Chip Product Ion (20.073 min) Frag=175.0V CID@5.2 (667.2268[z=1] -> **) GMO3aMSMS.d
325.1146
- Hex
- Hex
- Hex
163.0594
Hex
667.2307
487.1680
215.1316
505.2019
290.9800
362.3583
560.2984
Counts vs. Mass-to-Charge (m/z)


x10 3
+MS Chip Product Ion (20.463 min) Frag=175.0V CID@5.2 (667.2268[z=1] -> **) GMO3aMSMS.d
325.1123
Hex
- Hex
- Hex
163.0586
- Hex
487.1598
667.2178
505.2019
649.2239
204.0874
261.6329
358.4281
412.1714
542.4014
Counts vs. Mass-to-Charge (m/z)


x10 2
+MS Chip Product Ion (22.383 min) Frag=175.0V CID@5.2 (667.2285[z=1] -> **) GMO1aMSMS.d
325.1073
- Hex
487.1674
163.0608
Hex
- Hex
343.1117
- Hex
667.2517
615.9124
454.1509
413.4380
505.2019
188.3073
257.2587
Counts vs. Mass-to-Charge (m/z)

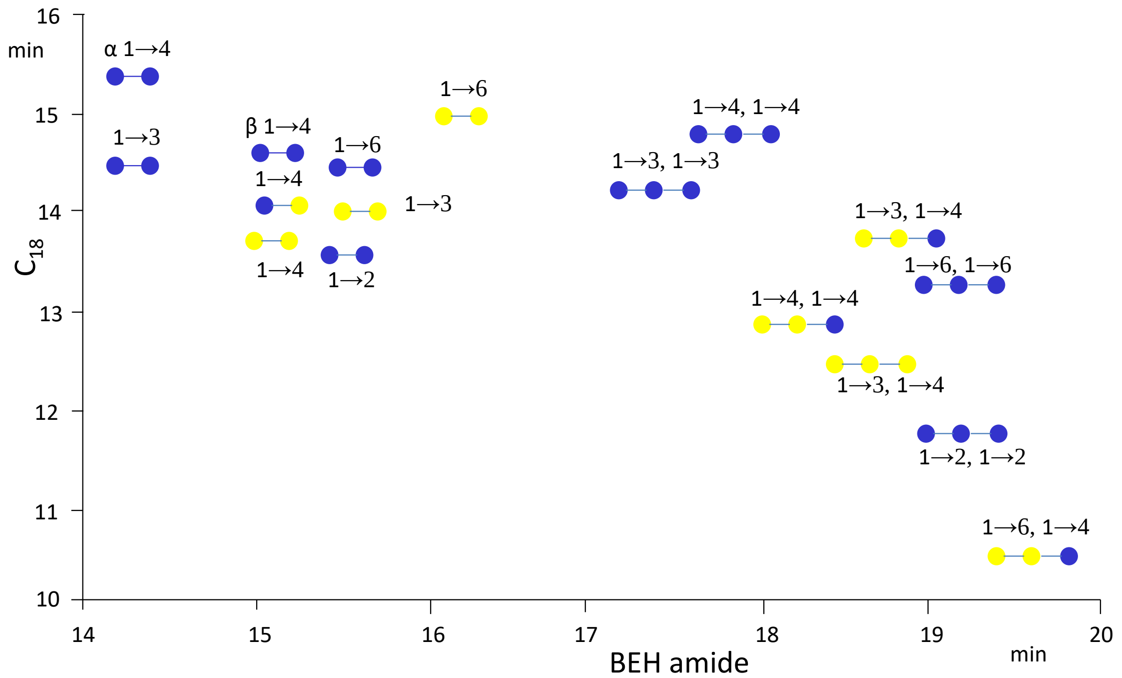
Anexo 5: Correspondiente a la sección 4.2

Separation of di- and trisaccharide mixtures by comprehensive two-dimensional liquid chromatography. Application to prebiotic oligosaccharides

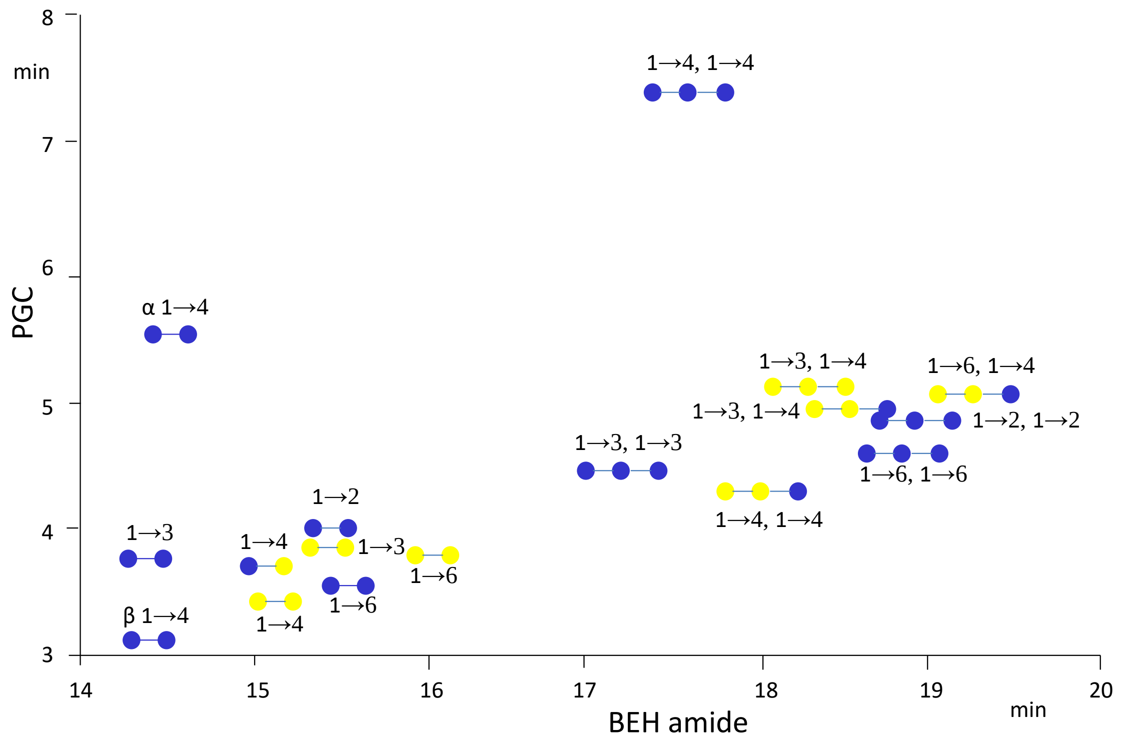
A. Martín Ortiz, A.I. Ruiz-Matute, M.L. Sanz, F.J. Moreno, M. Herrero

Analytica Chimica Acta, 2019, 1060, 125-132

Supplementary Material



16
min
α 1→4
15
1→3
β 1→4
1→6
1→4
1→6
1→4, 1→4
1→3, 1→3
14
1→3
C_{18}
1→4
1→2
1→3, 1→4
1→6, 1→6
13
1→4, 1→4
1→3, 1→4
12
1→2, 1→2
11
1→6, 1→4
10
14
15
16
17
18
19
20
BEH amide
min



8
min
1→4, 1→4
7
6
PGC
α 1→4
1→3, 1→4
1→6, 1→4
5
1→3, 1→4
1→2, 1→2
1→3, 1→3
1→6, 1→6
1→2
1→4, 1→4
4
1→3
1→4
1→3
1→6
1→6
1→4
β 1→4
3
14
15
16
17
18
19
20
BEH amide
min

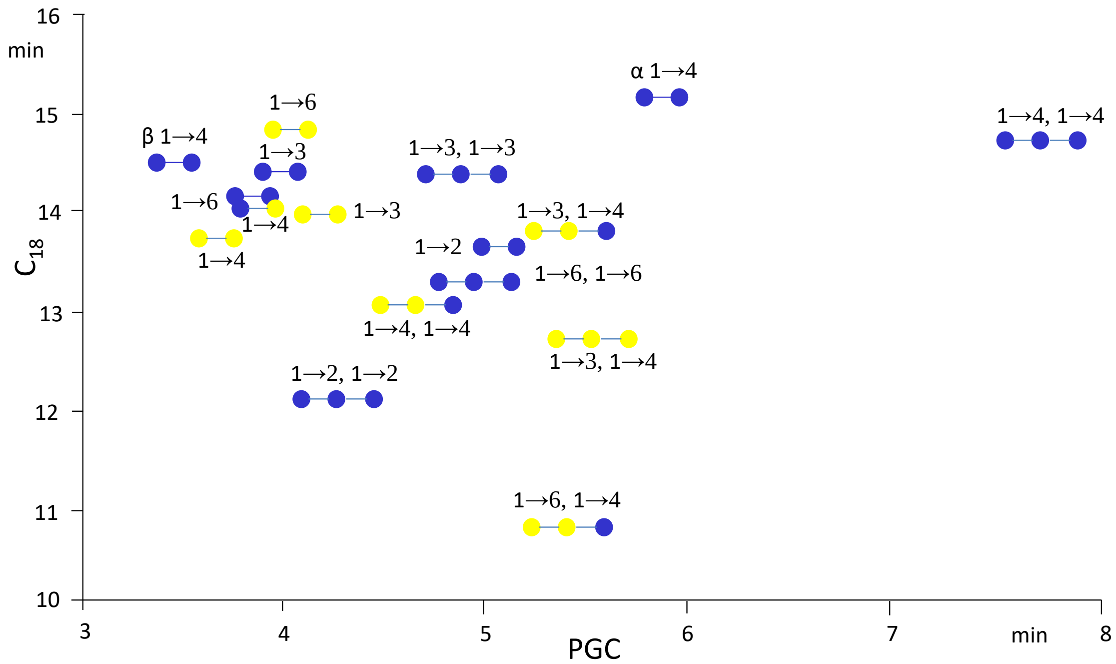


Figure S1: Reconstructed chromatograms for retention times of derivatized di- and trisaccharides (represented with the linkage and the different units) eluting on the three evaluated columns. ●Glucose unit, ●galactose unit

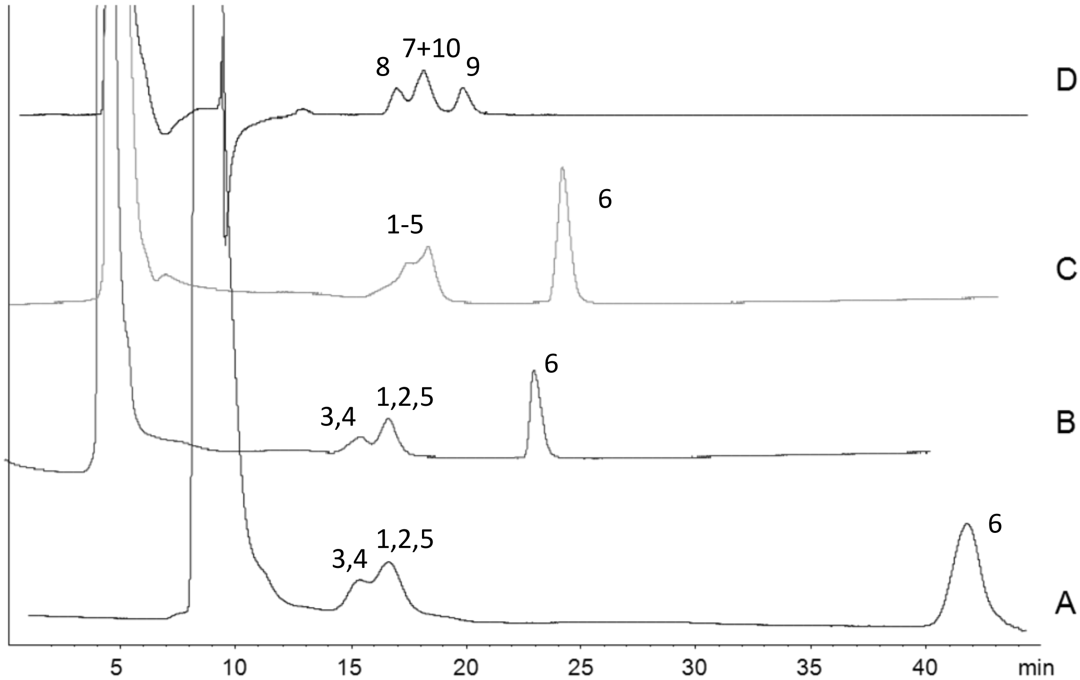


Figure S2. First dimension separation obtained under HILIC conditions of a mixture of kojibiose (1), cellobiose (2), laminaribiose (3), maltose (4), isomaltose (5) and kojitriose (6) using different gradients using acetonitrile (solvent A) and water (solvent B): A) 10% B to 50% B in 60 min; B) 10% B to 50% B in 30 min; and C) 10% B to 60% B in 40 min; and D) separation of a mixture of trisaccharide standards (3'-galactosyl-lactose (7), 4'-galactosyl-lactose (8), 6'-galactosyl-lactose (9) and galactotriose (10)) using the optimum ^{1}D gradient (A). Other analytical conditions: flow rate: 0.05 ml min^{-1}; V_{inj}: 3 µl; column: BEH Amide (150×2.1 mm, 3.5 µm).

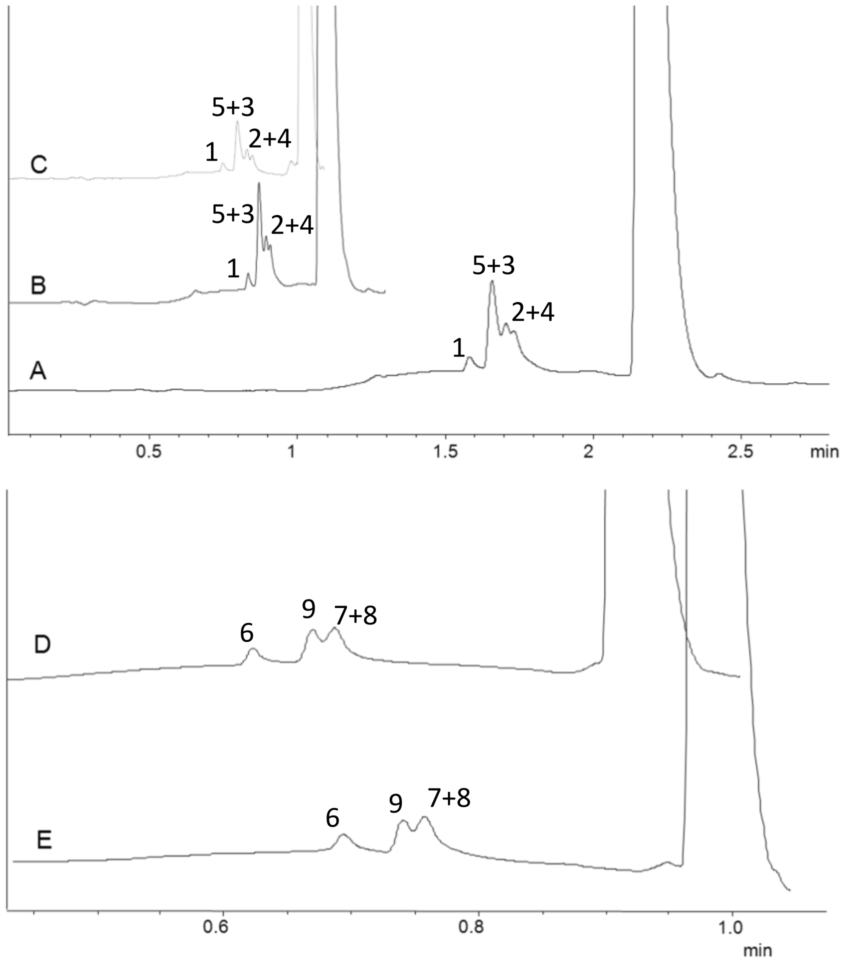


Figure S3. TOP: Second dimension separation (C_{18}, 30×4.6 mm, 2.7 µm) of kojibiose (1), cellobiose (2), laminaribiose (3), maltose (4) and isomaltose (5) obtained using optimum gradient profiles at different flow rates: A, 1.0 mL min^{-1}; B, 2.0 mL min^{-1}; C, 2.5 mL min^{-1}. DOWN: Second dimension separation of kojitriose (6), laminatriose (7), maltotriose (8) and isomaltotriose (9) attainable at 2.5 mL min^{-1} using 30 mm (D) and 50 mm (E) C_{18} partially porous columns. For other separation conditions, see text.

Anexo 6: Currículum Vitae

Lista de publicaciones

Publicaciones científicas incluidas en la tesis doctoral

- M.J. García-Sarrió, S. Rodríguez-Sánchez, A. Martín-Ortiz. Análisis De Carbohidratos Bioactivos Mediante Cromatografía De Interacción Hidrofílica (HILIC). *Cromatografía y Técnicas Afines, 2014, 35(2), 47-58.*
- A. Martín-Ortiz, J. Salcedo, D. Barile, A. Bunyatratchata, F.J. Moreno, I. Martin-García, A. Clemente, M.L. Sanz, A.I.Ruiz-Matute. Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry. *Journal of Chromatography A (Número especial), 2016, 1428, 143-153.*
- A. Martín-Ortiz, D. Barile, J. Salcedo, F.J. Moreno, A. Clemente , A.I. Ruiz-Matute, M.L. Sanz. Changes in caprine milk oligosaccharides at different lactation stages analyzed by high performance liquid chromatography coupled to mass spectrometry. *Journal of Agricultural and Food Chemistry, 2017, 65 (17) 3523-3531.*
- A. Martín-Ortiz, A.I. Ruiz Matute, M.L. Sanz, F.J. Moreno, M. Herrero. Separation of di- and trisaccharide mixtures by comprehensive two dimensional liquid chromatography. Application to prebiotic oligosaccharides, *Analytica Chimica Acta, 2019, 1060, 125-132.*
- A. Martín-Ortiz, F.J. Moreno, A.I. Ruiz Matute, M.L. Sanz. Selective Biotechnological Fractionation of Goat Milk Carbohydrates, *International Dairy Journal, 2019, 94, 38-45*
- A. Martín-Ortiz, O. Hernández-Hernández, C. Carrero-Carralero, R. Lebrón-Aguilar, A.I. Ruiz-Matute, F.J. Moreno, M.L. Sanz. Advances in structure elucidation of low molecular weight carbohydrates by liquid chromatography-tandem mass spectrometry analysis, *Journal of Chromatography B, 2019. (Enviado)*
- A. Martín-Ortiz, M.L. Sanz, F.J. Moreno, A.I. Ruiz-Matute. Development of a gas chromatographic-mass spectrometric method for the analysis of goat milk oligosaccharides. *(En redacción)*.

Otras publicaciones científicas

- A. Martín-Ortiz, Empleo de técnicas cromatográficas y espectrométricas para la caracterización de oligosacáridos bioactivos de leche. Cromatografía y Técnicas Afines, 2016, 37(2), 1-3.
- S. Rodríguez-Sánchez, A. Martín-Ortiz, S. Ramos, M.L. Sanz, A.C. Soria. Pressurized liquid extraction and chromatographic analysis of iminosugars from Aglaonema sp. leaves. Stability, toxicity and bioactivity of the extracts. Food Chemistry, 2016, 204, 62-69.

Participación en congresos

Comunicaciones orales (relacionadas con la tesis)

- A. Martín Ortiz, D. Barile, J. Salcedo, A. Clemente, F. J. Moreno, A. I. Ruiz-Matute, M. L. Sanz "Characterization of goat colostrum oligosaccharides by Nano-Liquid Chromatography on chip Quadrupole Time-of-Flight Mass Spectrometry and Hydrophilic Interaction Liquid Chromatography-Quadrupole Mass Spectrometry"

  15ª Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines, Castellón, 2015.
- A. Martín Ortiz "Nuevas metodologías basadas en LC-MS para el análisis de oligosacáridos bioactivos de leche de cabra". Jornada de desayunos científicos en el Instituto de Química Orgánica General (CSIC). 2016
- A. Martín Ortiz "Caracterización exhaustiva de oligosacáridos de calostro de cabra por Cromatografía de Líquidos acoplada a Espectrometría de Masas". I Simposio Anual en Química Avanzada, en la Facultad de Ciencias Químicas de la Universidad Complutense de Madrid. 2016.
- A. Martín Ortiz, D. Barile, J. Salcedo, A. Clemente, F. J. Moreno, A. I. Ruiz-Matute, M. L. Sanz "Comprehensive analysis of goat milk oligosaccharides"

  15ª Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines, Sevilla, 2016.

- A. Martín Ortiz, A.I. Ruiz Matute, M. L. Sanz, F. J. Moreno, M. Herrero, "Development of a new methodology for the analysis of bioactive oligosaccharides by comprehensive two-dimensional hydrophilic interaction×reversed phase liquid chromatography"

Premio 2º mejor comunicación oral en 18ª Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines, Granada, 2018.

Comunicaciones tipo póster (relacionadas con la tesis)

- A. Martín Ortiz, A. Clemente, F. J. Moreno, A. I. Ruiz-Matute, M. L. Sanz "Evaluation of goat milk oligosaccharides composition at different stages of the lactation period" Eurofoodchem XVIII, Madrid, 2015.

- A. Martín-Ortiz, R. Jimenez, A.I.Ruiz-Matute, F.J. Moreno, M.L. Sanz "Evaluation of an enzymatic treatment for th selective fractionation of goat milk carbohydrates prior to high performance liquid chromatographic analysis"

  HPLC 2017, Praga, República Checa, 2017.

- A. Martín Ortiz, M. L. Sanz, F. J. Moreno A. I. Ruiz-Matute "Development of a derivatization method for the analysis of goat milk oligosaccharids by gas chromatography-mass spectrometry".

  19th International Symposium on Advances in Extraction Technologies (Extech), Santiago de Compostela, España, 2017.

- A. Martín Ortiz, A.I. Ruiz Matute, M. L. Sanz, F. J. Moreno, M. Herrero "A novel methodology for the separation and analysis of bioactive oligosaccaharides by comprehensive two-dimensional liquid chromatography ".

  Premio mejor póster en III Jornadas Científicas Cial Fórum, Madrid, Noviembre 2018.

Otros congresos

- S. Rodriguez-Sanchez, A. Martín-Ortiz, C. Carrero-Carralero, S. Ramos, M.L. Sanz, A.C. Soria. "Pressurized liquid extraction of iminosugars from *Aglaonema* sp. Stability, toxicity and bioactivity of the extracts".

14ªs Jornadas de Análisis Instrumental (JAI), Barcelona, 2014.

- Miembro del comité organizador de la IX reunion de expertos en tecnologías de fluidos comprimidos (Flucomp). Madrid, 2018.

Premios

- Comité Científico del CIAL forum 2018 otorga el **primer premio a mejor póster** científico a A. Martín Ortiz, A.I. Ruiz Matute, M. L. Sanz, F. J. Moreno, M. Herrero, por su contribución con el trabajo titulado: "A novel methodology for the separation and analysis of bioactive oligosaccaharides by comprehensive two-dimensional liquid chromatography " presentado en las III Jornadas Científicas Cial Fórum celebrada en Madrid, 2018.
- XIV Edición de los Premios José Antonio García Domínguez otorga el **segundo premio a la mejor comunicación oral** a A. Martín Ortiz, A.I. Ruiz Matute, M. L. Sanz, F. J. Moreno, M. Herrero, por su contribución con el trabajo titulado: "Development of a new methodology for the analysis of bioactive oligosaccharides by comprehensive two-dimensional hydrophilic interaction×reversed phase liquid chromatography" presentado en las 18ª Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines, Granada, 2018.

Estancias en el extranjero

- Estancia en Robert Mondavi Institute. Department of Food and Science & Technology University of California, Davis, California, EEUU. 19/06/2015 – 15/08/2015
- Estancia en University of Reading. Department of Food and Nutritional Sciences. Reading, Inglaterra, Reino Unido. 16/05/2017 – 23/08/2017

Participación en contratos de investigación con empresas

- ACTIVIDAD: Synthesis of sweet functional oligosaccharides

  ENTIDAD FINANCIADORA: OPTIBIOTIX

  MODO DE PARTICIPACIÓN: Contrato de apoyo tecnológico

  ÁMBITO TERRITORIAL: Europa

  VIGENCIA: 2015-2017

  INVESTIGADOR/A PRINCIPAL: Dra. M.L. Sanz y Dr. F.J. Moreno

- ACTIVIDAD: Analysis and structural characterization of Vivinal GOS and Arla-GOS

  ENTIDAD FINANCIADORA: ARLAFOODS

  MODO DE PARTICIPACIÓN: Contrato de apoyo tecnológico

  ÁMBITO TERRITORIAL: Europa

  VIGENCIA: 2013-2014

  INVESTIGADOR/A PRINCIPAL: Dra. M.L. Sanz y Dr. F.J. Moreno